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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁵ : C12N 15/62, 15/56, 15/55 C12N 15/53, 1/19, 11/16 // (C12N 1/19, C12R 1:865)	A1	(11) International Publication Number: WO 94/01567 (43) International Publication Date: 20 January 1994 (20.01.94)
(21) International Application Number: PCT/EP93/01763 (22) International Filing Date: 7 July 1993 (07.07.93) (30) Priority data: 92202080.5 8 July 1992 (08.07.92) EP (34) Countries for which the regional or international application was filed: NL et al. 92203899.7 14 December 1992 (14.12.92) EP (34) Countries for which the regional or international application was filed: NL et al. (71) Applicant (for AU BB CA GB IE LK MN MW NZ SD only): UNILEVER PLC [GB/GB]; Unilever House, Blackfriars, London EC4 4BQ (GB).	(71) Applicant (for all designated States except AU BB CA GB IE LK MN MW NZ SD US): UNILEVER N.V. [NL/NL]; Weena 455, NL-3013 AL Rotterdam (NL). (72) Inventors; and (75) Inventors/Applicants (for US only): KLIS, Franciscus, Maria [NL/NL]; Benedenlangs 102, NL-1025 KL Amsterdam (NL). SCHREUDER, Maarten, Pleun [NL/NL]; Rode Kruislaan 1220, NL-1111 XB Diemen (NL). TOSCHKA, Holger, York [NL/NL]; Coornhertstraat 77, NL-3132 GB Vlaardingen (NL). VERRIPS, Cornelis, Theodorus [NL/NL]; Hagedoorn 18, NL-3142 KB Maassluis (NL). (74) Agent: VAN DER TOORREN, Johannes; Unilever N.V., Patent Division, P.O. Box 137, NL-3130 AC Vlaardingen (NL). (81) Designated States: AT, AU, BB, BG, BR, BY, CA, CH, CZ, DE, DK, ES, FI, GB, HU, JP, KP, KR, KZ, LK, LU, MG, MN, MW, NL, NO, NZ, PL, PT, RO, RU, SD, SE, SK, UA, US, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG). Published <i>With international search report.</i>	
(54) Title: PROCESS FOR IMMOBILIZING ENZYMES TO THE CELL WALL OF A MICROBIAL CELL BY PRODUCING A FUSION PROTEIN (57) Abstract A method is provided for immobilizing an enzyme, comprising immobilizing the enzyme or a functional part thereof to the cell wall of a microbial cell using recombinant DNA techniques. The enzyme is immobilized by linking it to the C-terminal part of a protein that ensures anchoring in the cell wall. Also provided is a recombinant polynucleotide comprising a structural gene encoding an enzyme protein, a part of a gene encoding the C-terminal part of a protein capable of anchoring in a eukaryotic or prokaryotic cell wall, as well as a signal sequence, in addition to a chimeric protein encoded by the recombinant polynucleotide and a vector and a microorganism containing the polynucleotide. The microorganism is suitable for carrying out enzymatic processes on an industrial scale.		

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PROCESS FOR IMMOBILIZING ENZYMES TO THE CELL WALL OF A MICROBIAL CELL BY PRODUCING A FUSION PROTEIN.

The present invention is in the field of conversion processes using immobilized
5 enzymes, produced by genetic engineering.

Background of the invention

In the detergent, personal care and food products industry there is a strong trend
towards natural ingredients of these products and to environmentally acceptable
10 production processes. Enzymic conversions are very important for fulfilling these
consumer demands, as these processes can be completely natural. Moreover enzymic
processes are very specific and consequently will produce minimum amounts of waste
products. Such processes can be carried out in water at mild temperatures and atmos-
pheric pressure. However enzymic processes based on free enzymes are either quite
15 expensive due to the loss of enzymes or require expensive equipment, like ultra-
membrane systems to entrap the enzyme.

Alternatively enzymes can be immobilized either physically or chemically. The latter
method has often the disadvantage that coupling is carried out using non-natural
chemicals and in processes that are not attractive from an environmental point of
20 view. Moreover chemical modification of enzymes is nearly always not very specific,
which means that coupling can affect the activity of the enzyme negatively.

Physical immobilization can comply with consumer demands, however also physical
immobilization may affect the activity of the enzyme in a negative way. Moreover, a
physically immobilized enzyme is in equilibrium with free enzyme, which means that
25 in continuous reactors, according to the laws of thermodynamics, substantial losses of
enzyme are unavoidable.

There are a few publications on immobilization of enzymes to microbial cells (see
reference 1). The present invention provides a method for immobilizing enzymes to
cell walls of microbial cells in a very precise way. Additionally, the immobilization
30 does not require any chemical or physical coupling step and is very efficient.

Some extracellular proteins are known to have special functions which they can
perform only if they remain bound to the cell wall of the host cell. Often this type of

protein has a long C-terminal part that anchors it in the cell wall. These C-terminal parts have very special amino acid sequences. A typical example is anchoring via C-terminal sequences enriched in proline (see reference 2). Another mechanism to anchor proteins in cell walls is that the protein has a glycosyl-phosphatidyl-inositol (GPI) anchor (see reference 3) and that the C-terminal part of the protein contains a substantial number of potential serine and threonine glycosylation sites. O-Glycosylation of these sites gives a rod-like conformation to the C-terminal part of these proteins. Another feature of these manno-proteins is that they seem to be linked to the glucan in the cell wall of lower eukaryotes, as they cannot be extracted from the cell wall with SDS, but can be liberated by glucanase treatment.

Summary of the invention

The present invention provides a method for immobilizing an enzyme, which comprises the use of recombinant DNA techniques for producing an enzyme or a functional part thereof linked to the cell wall of a host cell, preferably a microbial cell, and whereby the enzyme or functional fragment thereof is localized at the exterior of the cell wall. Preferably the enzyme or the functional part thereof is immobilized by linking to the C-terminal part of a protein that ensures anchoring in the cell wall.

In one embodiment of the invention a recombinant polynucleotide is provided comprising a structural gene encoding a protein providing catalytic activity and at least a part of a gene encoding a protein capable of anchoring in a eukaryotic or prokaryotic cell wall, said part encoding at least the C-terminal part of said anchoring protein. Preferably the polynucleotide further comprises a sequence encoding a signal peptide ensuring secretion of the expression product of the polynucleotide. Such signal peptide can be derived from a glycosyl-phosphatidyl-inositol (GPI) anchoring protein, α -factor, α -agglutinin, invertase or inulinase, α -amylase of *Bacillus*, or a proteinase of lactic acid bacteria. The DNA sequence encoding a protein capable of anchoring in the cell wall can encode α -agglutinin, AGA1, FLO1 or the Major Cell Wall Protein of lower eukaryotes, or a proteinase of lactic acid bacteria. The recombinant polynucleotide is operably linked to a promoter, preferably an inducible

promoter. The DNA sequence encoding a protein providing catalytic activity can encode a hydrolytic enzyme, e.g. a lipase, or an oxidoreductase, e.g. an oxidase.

Another embodiment of the invention relates to a recombinant vector comprising a polynucleotide as described above. If such vector contains a DNA sequence encoding
5 a protein providing catalytic activity, which protein exhibits said catalytic activity when present in a multimeric form, said vector can further comprise a second polynucleotide comprising a structural gene encoding the same protein providing catalytic activity combined with a sequence encoding a signal peptide ensuring secretion of the expression product of said second polynucleotide, said second
10 polynucleotide being operably linked to a regulatable promoter, preferably an inducible or repressible promoter.

A further embodiment of the invention relates to a chimeric protein encoded by a polynucleotide as described above.

Still another embodiment is a host cell, preferably a microorganism, containing a
15 polynucleotide as described above or a vector as described above. If the protein providing catalytic activity exhibits said catalytic activity when present in a multimeric form, said host cell or microorganism can further comprise a second polynucleotide comprising a structural gene encoding the same protein providing catalytic activity combined with a sequence encoding a signal peptide ensuring secretion of the
20 expression product of said second polynucleotide, said second polynucleotide being operably linked to a regulatable promoter, preferably an inducible or repressible promoter, and said second polynucleotide being present either in another vector or in the chromosome of said microorganism. Preferably the host cell or microorganism has at least one of said polynucleotides integrated in its chromosome. As a result of
25 culturing such host cell or microorganism the invention provides a host cell, preferably a microorganism, having a protein as described above immobilized on its cell wall. The host cell or microorganism can be a lower eukaryote, in particular a yeast.

The invention also provides a process for carrying out an enzymatic process by using
30 an immobilized catalytically active protein, wherein a substrate for said catalytically active protein is contacted with a host cell or microorganism according to the invention.

Brief Description of the Figures

Figure 1: DNA sequence of the 6057 bp *Hind*III fragment containing the complete AG α 1 gene of *S. cerevisiae* (see SEQ ID NO: 1 and 2). The position of the unique *Nhe*I site and the *Hind*III site used for the described constructions is specified in the header.

Figure 2: Schematic presentation of the construction of pUR2969. The restriction sites for endonucleases used are shown. Abbreviations used: AG-alpha-1: Gene expressing α -agglutinin from *S. cerevisiae*

amp: β -lactamase resistance gene

PGKp: phosphoglyceratekinase promoter

PGKt: terminator of the same gene.

Figure 3: α -Galactosidase activity of *S. cerevisiae* MT302/1C cells and culture fluid transformed with pSY13 during batch culture:

A: U/l α -galactosidase per time; the OD₅₃₀ is also shown

B: α -galactosidase activity of free and bond enzyme expressed in U/OD₅₃₀.

Figure 4: α -Galactosidase activity of *S. cerevisiae* MT302/1C cells and culture fluid transformed with pUR2969 during batch culture:

A: U/l α -galactosidase per time; the OD₅₃₀ is also shown

B: α -galactosidase activity of free and bond enzyme expressed in U/OD₅₃₀.

Figure 5: Western analysis with anti α -galactosidase serum of extracellular fractions of cells of exponential phase (OD₅₃₀=2). The analyzed fractions are equivalent to 4 mg cell walls, (fresh weight):

A: MT302/1C expressing α -galactosidase,

lane 1, growth medium

lane 2, SDS extract of isolated cell walls

lane 3, glucanase extract of SDS extracted cell walls;

B: MT302/1C expressing α -Gal-AG α 1 fusion protein,

lane 1, growth medium

lane 2, SDS extract of isolated cell walls

lane 3, glucanase extract of SDS-extracted cell walls

lane 4: Endo-H treated glucanase extract.

Figure 6: Immunofluorescent labelling (anti α -galactosidase) of MT302/1C cells in the exponential phase ($OD_{530}=2$) expressing the α -Gal- α -agglutinin fusion protein.

Phase micrograph of intact cells

A: overview

B: detail.

Figure 7: Schematic presentation of the construction of pUR2970A, pUR2971A,

- 5 pUR2972A, and pUR2973. The restriction sites for endonucleases used are indicated in the figure. PCR oligonucleotide sequences are mentioned in the text.

Abbreviations used: AGa1 cds: coding sequence of α -agglutinin

a-AGG=AGa1:

Gene expressing α -agglutinin from *S. cerevisiae*

amp: β -lactamase resistance gene

Pgal7=GAL7:

GAL7 promoter

- 10 lipolase: lipase gene of *Humicola*

invSS:

SUC2 signal sequence

a-MF: prepro- α -mating factor sequence

a-gal:

α -galactosidase gene

LEU2d: truncated promoter of *LEU2* gene;

LEU2: *LEU2* gene with complete promoter sequence.

- Figure 8: DNA sequence of a fragment containing the complete coding sequence of
15 lipase B of *Geotrichum candidum* strain 335426 (see SEQ ID NO: 11 and 12). The sequence of the mature lipase B starts at nucleotide 97 of the given sequence. The coding sequence starts at nucleotide 40 (ATG).

Figure 9: Schematic presentation of the construction of pUR2975 and pUR2976. The restriction sites for endonucleases used are shown. Abbreviations used:

- 20 a-AGG: Gene expressing α -agglutinin from *S. cerevisiae*

amp: β -lactamase resistance gene

Pgal7=GAL7:

GAL7 promoter

invSS: *SUC2* signal sequence

a-MF: prepro- α -mating factor sequence

LEU2d: truncated promoter *LEU2* gene

lipolase:

lipase gene of *Humicola*

lipaseB: lipase gene of *Geotrichum candidum*.

- 25 Figure 10: Schematic presentation of the construction of pUR2981 and pUR2982. The restriction sites for endonucleases used are shown. Abbreviations used:

a-AGG=AG- α 1: Gene expressing α -agglutinin from *S. cerevisiae*

mucor lipase: lipase gene of *Rhizomucor miehei*

2u:

2 μ m sequence

Pgal7=GAL7: *GAL7* promoter

invSS:

SUC2 signal sequence

- 30 a-MF: prepro- α -mating factor sequence

lipolase:

lipase gene of *Humicola*

amp: β -lactamase resistance gene;

LEU2d: truncated promoter *LEU2* gene

LEU2: *LEU2* gene with complete promoter sequence.

Figure 11: DNA sequence (2685 bases) of the 894 amino acids coding part of the *FLO1* gene (see SEQ ID NO: 21 and 22), the given sequence starts with the codon for the first amino acid and ends with the stop codon.

Figure 12: Schematic presentation of plasmid pUR2990. Some restriction sites for en-
5 donucleases relevant for the given cloning procedure are shown.

Figure 13: Schematic presentation of plasmid pUR7034.

Figure 14: Schematic presentation of plasmid pUR2972B.

Figure 15: Immunofluorescent labelling (anti-lipolase) of SU10 cells in the exponential phase ($OD_{530}=0.5$) expressing the lipolase/- α -agglutinin fusion protein.

10 A: phase micrograph B: matching fluorescent micrograph

Detailed description of the invention

The present invention provides a method for immobilizing an enzyme, comprising immobilizing the enzyme or a functional part thereof to the cell wall of a host cell,
15 preferably a microbial cell, using recombinant DNA techniques. In particular, the C-terminal part of a protein that ensures anchoring in the cell wall is linked to an enzyme or the functional part of an enzyme, in such a way that the enzyme is localized on or just above the cell surface. In this way immobilized enzymes are obtained on the surface of cells. The linkage is performed at gene level and is
20 characterized in that the structural gene coding for the enzyme is coupled to at least part of a gene encoding an anchor-protein in such a way that in the expression product the enzyme is coupled at its C-terminal end to the C-terminal part of an anchor-protein. The chimeric enzyme is preferably preceded by a signal sequence that ensures efficient secretion of the chimeric protein.

25 Thus the invention relates to a recombinant polynucleotide comprising a structural gene encoding a protein providing catalytic activity and at least a part of a gene encoding a protein capable of anchoring in a eukaryotic or prokaryotic cell wall, said part encoding at least the C-terminal part of said anchoring protein. The length of the C-terminal part of the anchoring protein may vary. Although the entire structural
30 protein could be used, it is preferred that only a part is used, leading to a more efficient exposure of the enzyme protein in the medium surrounding the cell. The

anchoring part of the anchoring protein should preferably be entirely present. As an example, about the C-terminal half of the anchoring protein could be used.

Preferably, the polynucleotide further comprises a sequence encoding a signal peptide ensuring secretion of the expression product of the polynucleotide. The signal peptide

5 can be derived from a GPI anchoring protein, α -factor, α -agglutinin, invertase or inulinase, α -amylase of *Bacillus*, or a proteinase of lactic acid bacteria.

The protein capable of anchoring in the cell wall is preferably selected from the group of α -agglutinin, AGA1, FLO1 (flocculation protein) or the Major Cell Wall Protein of lower eukaryotes, or a proteinase of lactic acid bacteria. The

10 polynucleotide of the invention is preferably operably linked to a promoter, preferably a regulatable promoter, especially an inducible promoter.

The invention also relates to a recombinant vector containing the polynucleotide as described above, and to a host cell containing this polynucleotide, or this vector.

In a particular case, wherein the protein providing catalytic activity exhibits said

15 catalytic activity when present in a multimeric form, such as may be the case with oxidoreductases, dimerisation or multimerisation of the monomers might be a prerequisite for activity. The vector and/or the host cell can then further comprise a second polynucleotide comprising a structural gene encoding the same protein providing catalytic activity combined with a sequence encoding a signal peptide ensuring
20 secretion of the expression product of said second polynucleotide, said second polynucleotide being operably linked to a regulatable promoter, preferably an inducible or repressible promoter. Expression and secretion of the second polynucleotide after expression and secretion of the first polynucleotide will then result in the formation of an active multimer on the exterior of the cell wall.

25 The host cell or microorganism preferably contains the polynucleotide described above, or at least one of said polynucleotides in the case of a combination, integrated in its chromosome.

The present invention relates in particular to lower eukaryotes like yeasts that have very stable cell walls and have proteins that are known to be anchored in the cell
30 wall, e.g. α -agglutinin or the product of gene *FLO1*. Suitable yeasts belong to the genera *Candida*, *Debaryomyces*, *Hansenula*, *Kluyveromyces*, *Pichia* and *Saccharomyces*.

Also fungi, especially *Aspergillus*, *Penicillium* and *Rhizopus* can be used. For certain applications also prokaryotes are applicable.

For yeasts the present invention deals in particular with genes encoding chimeric enzymes consisting of:

- 5 a. the signal sequence e.g. derived from the α -factor-, the invertase-, the α -agglutinin- or the inulinase genes;
- b. structural genes encoding hydrolytic enzymes such as α -galactosidase, proteases, peptidases, pectinases, pectylesterase, rhamnogalacturonase, esterases and lipases, or non-hydrolytic enzymes such as oxidases; and
- 10 c. the C-terminus of typically cell wall bound proteins such as α -agglutinin (see reference 4), AGA1 (see reference 5) and FLO1 (see the non-prior published reference 6).

The expression of these genes can be under the control of a constitutive promoter, but more preferred are regulatable, i.e. repressible or inducible promoters such as the
15 *GAL7* promoter for *Saccharomyces*, or the inulinase promoter for *Kluyveromyces* or the methanol-oxidase promoter for *Hansenula*.

Preferably the constructs are made in such a way that the new genetic information is integrated in a stable way in the chromosome of the host cell.

The invention further relates to a host cell, in particular a microorganism, having the
20 chimeric protein described above immobilized on its cell wall. It further concerns the use of such microorganisms for carrying out an enzymatic process by contacting a substrate for the enzyme with the microorganism. Such a process may be carried out e.g. in a packed column, wherein the microorganisms may be supported on solid particles, or in a stirred reactor. The reaction may be aqueous or non-aqueous. Where
25 necessary, additives necessary for the performance of the enzyme, e.g. a co-factor, may be introduced in the reaction medium.

After repeated usage of the naturally immobilized enzyme system in processes, the performance of the system may decrease. This is caused either by physical denaturation or by chemical poisoning or detachment of the enzyme. A particular
30 feature of the present invention is that after usage the system can be recovered from the reaction medium by simple centrifugation or membrane filtration techniques and that the thus collected cells can be transferred to a recovery medium in which the

cells revive quickly and concomitantly produce the chimeric protein, thus ensuring that the surface of the cells will be covered by fully active immobilized enzyme. This regeneration process is simple and cheap and therefore will improve the economics of enzymic processes and may result in a much wider application of processes based on
5 immobilized enzyme systems.

However, by no means the present invention is restricted to the reusability of the immobilized enzymes.

The invention will be illustrated by the following examples without the scope of the invention being limited thereto.

10

EXAMPLE 1 Immobilized α -galactosidase/ α -agglutinin on the surface of *S. cerevisiae*.

The gene encoding α -agglutinin has been described by Lipke *et al.* (see reference 4). The sequence of a 6057 bp *Hind*III insert in pTZ18R, containing the whole AG α 1
15 gene is given in Figure 1. The coding sequence expands over 650 amino acids, including a putative signal sequence starting at nucleotide 3653 with ATG. The unique *Nhe*I site cuts the DNA at position 988 of the given coding sequence within the coding part of amino acid 330, thereby separating the α -agglutinin into an N-terminal and a C-terminal part of about same size.

20 Through digestion of pUR2968 (see Figure 2) with *Nhe*I/*Hind*III a 1.4 kb fragment was released, containing the sequence information of the putative cell wall anchor. For the fusion to α -galactosidase the plasmid pSY16 was used, an episomal vector based on YEplac 181, harbouring the α -galactosidase sequence preceded by the *SUC*2 invertase signal sequence and placed between the constitutive *PGK* promoter and
25 *PGK* terminator. The *Sty*I site, present in the last nine base-pairs of the open reading frame of the α -galactosidase gene, was ligated to the *Nhe*I site of the AG α 1 gene fragment. To ensure the in frame fusion, the *Sty*I site was filled in and the 5' overhang of the *Nhe*I site was removed, prior to ligation into the *Sty*I/ *Hind*III digested pSY13 (see Figure 2).

30 To verify the correct assembly of the new plasmid, the shuttle vector was transformed into *E. coli* JM109 (*recA1 supE44 endA1 hsdR17 gyrA96 relA1 thi* Δ (*lac-proAB*) F' [*traD36 proAB*⁺ *lacI*^s *lacZ* Δ M15]) (see reference 7) by the transformation protocol

described by Chung *et al.* (see reference 8). One of the positive clones, designated pUR2969, was further characterized, the DNA isolated and purified according to the Quiagen protocol and subsequently characterized by DNA sequencing. DNA sequencing was mainly performed as described by Sanger *et al.* (see reference 9), and

5 Hsiao (see reference 10), here with the Sequenase version 2.0 kit from United States Biochemical Company, according to the protocol with T7 DNA polymerase (Amersham International plc) and [³⁵S]dATP α S (Amersham International plc: 370 MBq/ml; 22 TBq/mmol).

This plasmid was then transformed into *S. cerevisiae* strain MT302/1C according to

10 the protocol from Klebe *et al.* (see reference 11).

Yeast transformants were selected on selective plates, lacking leucine, on with 40 μ l (20mg/ml DMF). X- α -Gal (5-bromo-4-chloro-3-indolyl- α -D-glucose, Boehringer Mannheim) was spread, to directly test for α -galactosidase activity (see reference 12). To demonstrate the expression, secretion, localization and activity of the chimeric

15 protein the following analyses were performed:

1. Expression and secretion

S. cerevisiae strain MT302/1C was transformed with either plasmid pSY13 containing the α -galactosidase gene of *Cyamopsis tetragonoloba* or plasmid pUR2969 containing the α -galactosidase/ α -agglutinin fusion construct. During batch culture α -galactosidase

20 activities were determined for washed cells and growth medium. The results are given in Figure 3 and Figure 4. The α -galactosidase expressed from yeast cells containing plasmid pSY13 was almost exclusively present in the growth medium (Figure 3A), whereas the α -galactosidase- α -agglutinin fusion protein was almost exclusively cell associated (Figure 4A). Moreover, the immobilized, cell wall-associated, α -galacto-

25 sidase- α -agglutinin fusion enzyme had retained the complete activity over the whole incubation time, while the secreted and released enzyme lost about 90% of the activity after an incubation of 65 hours. This indicates, that the immobilization of the described enzyme into the cell wall of yeast protects the enzyme against inactivation, presumably through proteinases, and thereby increases the stability significantly.

30 Further insight into the localization of the different gene products was obtained by Western analysis. Therefore, cells were harvested by centrifugation and washed in 10 mM Tris.HCl, pH 7.8; 1 mM PMSF at 0°C and all subsequent steps were performed

at the same temperature. Three ml isolation buffer and 10 g of glass beads were added per gram of cells (wet weight). The mixture was shaken in a Griffin shaker at 50% of its maximum speed for 30 minutes. The supernatant was isolated and the glass beads were washed with 1 M NaCl and 1 mM PMSF until the washes were clear. The supernatant and the washes were pooled. The cell walls were recovered by centrifugation and were subsequently washed in 1 mM PMSF.

Non-covalently bound proteins or proteins bound through disulphide bridges were released from cell walls by boiling for 5 minutes in 50 mM Tris.HCl, pH 7.8; containing 2 % SDS, 100 mM EDTA and 40 mM β -mercaptoethanol. The SDS-extracted cell walls were washed several times in 1 mM PMSF to remove SDS. Ten mg of cell walls (wet weight) were taken up in 20 l 100 mM sodium acetate, pH 5.0, containing 1 mM PMSF. To this, 0.5 mU of the β -1,3-glucanase (Laminarase; Sigma L5144) was used as a source of β -1,3-glucanase) was added followed by incubation for 2 hours at 37 °C. Subsequently another 0.5 mU of β -1,3-glucanase was added, followed by incubation for another 2 hours at 37 °C.

Proteins were denatured by boiling for 5 minutes preceding Endo-H treatment. Two mg of protein were incubated in 1 ml 50 mM potassium phosphate, pH 5.5, containing 100 mM β -mercaptoethanol and 0.5 mM PMSF with 40 mU Endo-H (Boehringer) for 48 hours at 37 °C. Subsequently 20 mU Endo-H were added followed by 24 hours of incubation at 37 °C.

Proteins were separated by SDS-PAGE according to Laemmli (see reference 13) in 2.2.-20% gradient gels. The gels were blotted by electrophoretic transfer onto Immobilon polyvinylidene-difluoride membrane (Millipore) as described by Towbin *et al.* (see reference 14). In case of highly glycosylated proteins a subsequently mild periodate treatment was performed in 50 mM periodic acid, 100 mM sodium acetate, pH 4.5, for several hours at 4 °C. All subsequent incubations were carried out at room temperature. The blot was blocked in PBS, containing 0.5% gelatine and 0.5% Tween-20, for one hour followed by incubation for 1 hour in probe buffer (PBS, 0.2% gelatine, 0.1% Tween-20) containing 1:200 diluted serum. The blot was subsequently washed several times in washing buffer (PBS; 0.2% gelatine; 0.5% Tween-20) followed by incubation for 1 hour in probe-buffer containing 125 I-labelled protein A

(Amersham). After several washes in washing buffer, the blot was air-dried, wrapped in Saran (Dow) and exposed to X-omat S film (Kodak) with intensifying screen at -70 °C. An Omnimedia 6cx scanner and the Adobe Photoshop programme were used to quantify the amount of labelled protein. The results of the various protein isolation
5 procedures from both transformants are given in Figure 5. While for the transformants comprising the pSY13 plasmid the overall mass of the enzyme was localized in the medium, with only minor amounts of enzyme more entrapped than bond in the cell wall (Figure 5A) -which could completely be removed by SDS extraction- the fusion protein was tightly bound to the cell wall; with only small amounts of
10 α -galactosidase/ α -agglutinin delivered into the surrounding culture fluid or being SDS extractable. In contrast to the laminarinase extraction of cell walls from cells expressing the free α -galactosidase, where no further liberation of any more enzyme was observed, identical treatment of fusion enzyme expressing cells released the overall bulk of the enzyme. This indicates that the fusion protein is intimately
15 associated with the cell wall glucan in *S. cerevisiae*, like α -agglutinin, while α -galactosidase alone is not. The subsequently performed EndoH treatment showed a heavy glycosylation of the fusion protein, a result, entirely in agreement with the described extended glycosylation of the C-terminal part of α -agglutinin.

2. Localization

20 Immunofluorescent labelling with anti- α -galactosidase serum was performed on intact cells to determine the presence and distribution of α -galactosidase/ α -agglutinin fusion protein in the cell wall. Immunofluorescent labelling was carried out without fixing according to Watzele *et al.* (see reference 15). Cells of OD₅₃₀=2 were isolated and washed in TBS (10 mM Tris.HCl, pH 7.8, containing 140 mM NaCl, 5 mM EDTA
25 and 20 μ g/ml cycloheximide). The cells were incubated in TBS + anti- α -galactosidase serum for 1 hour, followed by several washings in TBS. A subsequent incubation was carried out with FITC-conjugated anti-rabbit IgG (Sigma) for 30 minutes. After washing in TBS, cells were taken up in 10 mM Tris.HCl, pH 9.0, containing 1 mg/ml p-phenylenediamine and 0.1 % azide and were photographed on a Zeiss 68000
30 microscope. The results of these analysis are given in Figure 6, showing clearly that the chimeric α -galactosidase/ α -agglutinin is localized at the surface of the yeast cell. Buds of various sizes, even very small ones very uniformly labelled, demonstrates that

the fusion enzyme is continuously incorporated into the cell wall throughout the cell cycle and that it instantly becomes tightly linked.

3. Activity

- To quantitatively assay α -galactosidase activity, 200 μ l samples containing 0.1 M sodium-acetate, pH 4.5 and 10 mM p-nitrophenyl- α -D-galactopyranoside (Sigma) were incubated at 37 °C for exactly 5 minutes. The reaction was stopped by addition of 1 ml 2% sodium carbonate. From intact cells and cell walls, removed by centrifugation and isolated and washed as described, the α -galactosidase activity was calculated using the extinction coefficient of p-nitrophenol of 18.4 cm²/mole at 410 nm.
- One unit was defined as the hydrolysis of 1 μ mole substrate per minute at 37 °C.

Table 1. Distribution of free and immobilized α -galactosidase activity in yeast cells

	<u>α-Galactosidase activity (U/g F.W. cells)</u>		
	Growth medium	Intact cells	Isolated cell walls
Expressed protein			
α -galactosidase	14.7	0.37	0.01
α Gal/ α AGG fusion protein	0.54	13.3	10.9

- Transformed MT302/1C cells were in exponential phase ($OD_{530}=2$). One unit is defined as the hydrolysis of 1 μ mole of p-nitrophenyl- α -D-galactopyranoside per minute at 37 °C.
- The results are summarized in Table 1. While the overall majority of α -galactosidase was distributed in the culture fluid, most of the fusion product was associated with the cells, primarily with the cell wall. Taking together the results shown in Figures 3 to 6 and in Table 1, it could be calculated that the enzymatic α -galactosidase activity of the chimeric enzyme is as good as that of the free enzyme. Moreover, during stationary phase, the activity of the α -galactosidase in the growth medium decreased, whereas the activity of the cell wall associated α -galactosidase α -agglutinin fusion

remained constant, indicating that the cell associated fusion protein is protected from inactivation or proteolytic degradation.

N.B. The essence of this EXAMPLE was published during the priority year by M.P.

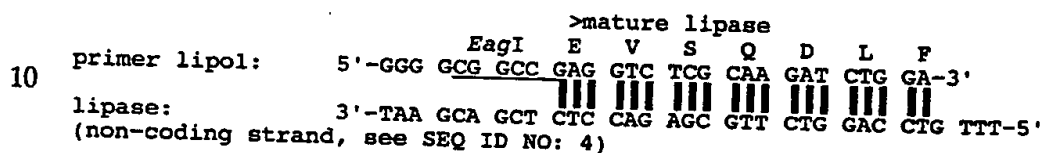
5 Schreuder *et al.* (see reference 25).

EXAMPLE 2A Immobilized *Humicola* lipase/ α -agglutinin on the surface of *S. cerevisiae*. (inducible expression of immobilized enzyme system)

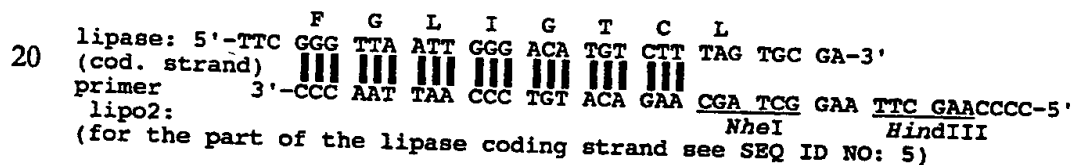
The construction and isolation of the 1.4 kb *NheI/HindIII* fragment containing the C-terminal part of α -agglutinin has been described in EXAMPLE 1. Plasmid pUR7021
10 contains an 894 bp long synthetically produced DNA fragment encoding the lipase of *Humicola* (see reference 16 and SEQ ID NO: 7 and 8), cloned into the *EcoRI/HindIII* restriction sites of the commercially available vector pTZ18R (see Figure 7). For the proper one-step modification of both the 5' end and the 3' end of
15 the DNA part coding for the mature lipase, the PCR technique can be applied. Therefore the DNA oligonucleotides lipo1 (see SEQ ID NO: 3) and lipo2 (see SEQ ID NO: 6) can be used as primers in a standard PCR protocol, generating an 826 bp long DNA fragment with an *EagI* and a *HindIII* restriction site at the ends, which can
20 be combined with the larger part of the *EagI/HindIII* digested pUR2650, a plasmid containing the α -galactosidase gene preceded by the invertase signal sequence as described earlier in this specification, thereby generating plasmid pUR2970A (see Figure 7).

PCR oligonucleotides for the in-frame linkage of *Humicola* lipase and the C-terminus of α agglutinin.

- 5 a: PCR oligonucleotides for the transition between *SUC2* signal sequence and the N-terminus of lipase.



- 15 b: PCR oligonucleotides for the in frame transition between C-terminus of lipase and C-terminal part of α -agglutinin.



- 25 Through the PCR method a *NheI* site will be created at the end of the coding sequence of the lipase, allowing the in-frame linkage between the DNA coding for lipase and the DNA coding for the C-terminal part of α -agglutinin. Plasmid pUR2970A can then be digested with *NheI* and *HindIII* and the 1.4 kb *NheI/HindIII* fragment containing the C-terminal part of α -agglutinin from plasmid pUR2968 can
 30 be combined with the larger part of *NheI* and *HindIII* treated plasmid pUR2970A, resulting in plasmid pUR2971A. From this plasmid the 2.2 kb *EagI/HindIII* fragment can be isolated and ligated into the *EagI*- and *HindIII*-treated pUR2741, whereby plasmid pUR2741 is a derivative of pUR2740 (see reference 17), where the second *EagI* restriction site in the already inactive *Tet* resistance gene was deleted through
 35 *NruI/SaII* digestion. The *SaII* site was filled in prior to religation. The ligation then results in pUR2972A containing the *GAL7* promoter, the invertase signal sequence, the chimeric lipase/ α -agglutinin gene, the 2 μ m sequence, the defective *Leu2* promoter and the *Leu2* gene. This plasmid can be used for transforming *S. cerevisiae* and the transformed cells can be cultivated in YP medium containing galactose as an
 40 inducer without repressing amounts of glucose being present, which causes the expression of the chimeric lipase/ α -agglutinin gene.

The expression, secretion, localization and activity of the chimeric lipase/ α -agglutinin can be analyzed using similar procedures as given in EXAMPLE 1.

In a similar way variants of *Humicola* lipase, obtained via rDNA techniques, can be
5 linked to the C-terminal part of α -agglutinin, which variants can have a higher stability during (inter)esterification processes.

EXAMPLE 2B Immobilized *Humicola* lipase/ α -agglutinin on the surface of *S. cerevisiae* (inducible expression of immobilized enzyme system)

10 **EXAMPLE 2A** describes a protocol for preparing a particular construct. Before carrying out the work it was considered more convenient to use the expression vector described in EXAMPLE 1, so that the construction route given in this EXAMPLE 2B differs on minor points from the construction route given in EXAMPLE 2A and the resulting plasmids are not identical to those described in EXAMPLE 2A. However,
15 the essential gene construct comprising the promoter, signal sequence, and the structural gene encoding the fusion protein are the same in EXAMPLES 2A and 2B.

1. Construction

The construction and isolation of the 1.4 kb *NheI/HindIII* fragment encoding the C-
20 terminal part of α -agglutinin cell wall protein has been described in EXAMPLE 1. The plasmid pUR7033 (resembling pUR7021 of EXAMPLE 2A) was made by treating the commercially available vector pTZ18R with *EcoRI* and *HindIII* and ligating the resulting vector fragment with an 894 bp long synthetically produced DNA *EcoRI/HindIII* fragment encoding the lipase of *Humicola* (see SEQ ID NO: 7
25 and 8, and reference 16).

For the fusion of the lipase to the C-terminal, cell wall anchor-comprising domain of α -agglutinin, plasmid pUR7033 was digested with *EagI* and *HindIII*, and the lipase coding sequence was isolated and ligated into the *EagI*- and *HindIII*-digested yeast expression vector pSY1 (see reference 27), thereby generating pUR7034 (see Figure
30 13). This is a 2 μ m episomal expression vector, containing the α -galactosidase gene described in EXAMPLE 1, preceded by the invertase (*SUC2*) signal sequence under the control of the inducible *GAL7* promoter.

Parallel to this digestion, pUR7033 was also digested with *EcoRV* and *HindIII*, thereby releasing a 57 bp long DNA fragment, possessing codons for the last 15 carboxyterminal amino acids. This fragment was exchanged against a small DNA fragment, generated through the hybridisation of the two chemically synthesized
5 deoxyoligonucleotides SEQ ID NO: 9 and SEQ ID NO: 10. After annealing of both DNA strands, these two oligonucleotides essentially reconstruct the rest of the 3' coding sequence of the initial lipase gene, but additionally introduce downstream of the lipase gene a new *NheI* restriction site, followed by a *HindIII* site in close vicinity, whereby the first three nucleotides of the *NheI* site form the codon for the last amino
10 acid of the lipase. The resulting plasmid was designated pUR2970B. Subsequently, this construction intermediate was digested with *EagI* and *NheI*, the lipase encoding fragment was isolated, and, together with the 1.4 kb *NheI/HindIII* fragment of pUR2968 ligated into the *EagI*- and *HindIII*-cut pSY1 vector. The outcome of this 3-point-ligation was called pUR2972B (see Figure 14), the final lipolase- α -agglutinin
15 yeast expression vector.

This plasmid was used for transforming *S. cerevisiae* strain SU10 as described in reference 17 and the transformed cells were cultivated in YP medium containing galactose as the inducer without repressing amounts of glucose being present, which causes the expression of the chimeric lipase/ α -agglutinin gene.

20 2. Activity

To quantify the lipase activity, two activity measurements with two separate substrates were performed. In both cases, SU10 yeast cells transformed with either plasmid pUR7034 or pSY1 served as control. Therefore, yeast cell transformants containing either plasmid pSY1 or plasmid pUR7034 or plasmid pUR2972B were grown up for
25 24h in YNB-glucose medium supplied with histidine and uracil, then diluted 1:10 in YP-medium supplied with 5% galactose, and again cultured. After 24h incubation at 30°C, a first measurement for both assays was performed.

The first assay applied was the pH stat method. Within this assay, one unit of lipase activity is defined as the amount of enzyme capable of liberating one micromole of
30 fatty acid per minute from a triglyceride substrate under standard assay conditions (30 ml assay solution containing 38 mM olive oil, considered as pure trioleate, emulsified with 1:1 w/w gum arabic, 20 mM calcium chloride, 40 mM sodium chloride, 5 mM

Tris, pH 9.0, 30°C) in a radiometer pH stat apparatus (pHM 84 pH meter, ABU 80 autoburette, TTA 60 titration assembly). The fatty acids formed were titrated with 0.05 N NaOH and the activity measured was based on alkali consumption in the interval between 1 and 2 minutes after addition of putative enzyme batch. To test for
 5 immobilized lipase activity, 1 ml of each culture was centrifuged, the supernatant was saved, the pellet was resuspended and washed in 1 ml 1 M sorbitol, subsequently again centrifuged and resuspended in 200µl 1 M sorbitol. From each type of yeast cell the first supernatant and the washed cells were tested for lipase activity.

10 A: Lipase activity after 24h (LU/ml)

	cell bound	culture fluid
pSY1	5.9	8.8
pUR7034	24.1	632.0
pUR2972B-(1)	18.7	59.6
15 pUR2972B-(2)	24.6	40.5

B: Lipase activity after 48h (LU/ml)

	cell bound	culture fluid	OD660
pSY1	6.4	4.3	~40
20 pUR7034	215.0	2750.0	~40
pUR2972B-(1)	37.0	87.0	~40
pUR2972B-(2)	34.0	82.0	~40

The rest of the yeast cultures was further incubated, and essentially the same
 25 separation procedure was done after 48 hours. Dependent on the initial activity measured, the actual volume of the sample measured deviated between 25µl and 150µl.

This series of measurements indicates, that yeast cells comprising the plasmid coding for the lipase- α -agglutinin fusion protein in fact express some lipase activity which is
 30 associated with the yeast cell.

An additional second assay was performed to further confirm the immobilization of activity of lipase on the yeast cell surface. Briefly, within this assay, the kinetics of the PNP (=paranitrophenyl) release from PNP-butyrate is determined by measurement of the OD at 400 nm. Therefore, 10 ml cultures containing yeast cells with either pSY1, pUR7034 or pUR2972B were centrifuged, the pellet was resuspended in 4 ml of buffer A (0.1 M NaOAc, pH 5.0 and 1 mM PMSF), from this 4 ml 500µl was centrifuged again and resuspended in 500 µl PNB-buffer (20 mM Tris-HCl, pH 9.0, 20 mM CaCl₂, 25 mM NaCl), centrifuged once again, and finally resuspended in 400µl PNB buffer. This fraction was used to determine the cell bound fraction of lipase.

The remaining 3500µl were spun down, the pellet was resuspended in 4 ml A, to each of this, 40µl laminarinase (ex mollusc, 1.25 mU/µl) was added and first incubated for 3 hours at 37°C, followed by an overnight incubation at 20°C. Then the reaction mixture, still containing intact cells, were centrifuged again and the supernatant was used to determined the amount of originally cell wall bound material released through laminarinase incubation. The final pellet was resuspended in 400µl PNP buffer, to calculate the still cell associated part. The blank reaction of a defined volume of specific culture fraction in 4 ml assay buffer was determined, and than the reaction was started through addition of 80µl of substrate solution (100 mM PNP-butyrate in methanol), and the reaction was observed at 25°C at 400 nm in a spectrophotometer.

	cell bound activity*	activity in the medium	laminarinase extract	laminarinase extracted cells	OD660
25 pSY1	0.001 (116µl)	0.001	0.028	0.000	2.6
pUR7034	0.293 (220µl)	0.446	0.076	0.985	2.36
pUR2972B-(1)	0.494 (143µl)	0.021	0.170	0.208	2.10

* unless otherwise mentioned, the volume of enzyme solution added was 20µl

This result positively demonstrates that a significant amount of lipase activity is immobilized on the surface yeast cell, containing plasmid pUR2972B. Here again,

incorporation took place in such a way, that the reaction was catalyzed by cell wall inserted lipase of intact cells, indicated into the exterior orientated immobilization. Furthermore, the release of a significant amount of lipase activity after incubation with laminarinase again demonstrates the presumably covalent incorporation of a heterologous enzyme through gene fusion with the C-terminal part of α -agglutinin.

3. Localization

The expression, secretion, and subsequent incorporation of the lipase- α -agglutinin fusion protein into the yeast cell wall was also confirmed through immunofluorescent labelling with anti-lipolase serum essentially as described in EXAMPLE 1, item

2. Localization.

As can be seen in Figure 15, the immunofluorescent stain shows essentially an analogous picture as the α -galactosidase immuno stain, with clearly detectable reactivity on the outside of the cell surface (see Figure 15 A showing a clear halo around the cells and Figure B showing a lighter circle at the surface of the cells), but neither in the medium nor in the interior of the cells. Yeast cells expressing pUR2972B, the *Humicola* lipase- α -agglutinin fusion protein, become homogeneously stained on the surface, indicating the virtually entire immobilization of a chimeric enzyme with an α -agglutinin C-terminus on the exterior of a yeast cell. In the performed control experiment SU10 yeast cells containing plasmid pUR7034 served as a control and here, no cell surface bound reactivity against the applied anti-lipase serum could be detected.

In a similar way variants of *Humicola* lipase, obtained via rDNA techniques, can be linked to the C-terminal part of α -agglutinin, which variants can have a higher stability during (inter)esterification processes.

EXAMPLE 3 Immobilized *Humicola* lipase/ α -agglutinin on the surface of *S. cerevisiae* (constitutive expression of immobilized enzyme system)

Plasmid pUR2972 as described in EXAMPLE 2 can be treated with *EagI* and *HindIII* and the about 2.2 kb fragment containing the lipase/ α -agglutinin gene can be isolated. Plasmid pSY16 can be restricted with *EagI* and *HindIII* and between these sites the 2.2 kb fragment containing the lipase/ α -agglutinin fragment can be ligated resulting in pUR2973. The part of this plasmid that is involved in the production of

the chimeric enzyme is similar to pUR2972 with the exception of the signal sequence. Whereas pUR2972 contains the *SUC2*-invertase-signal sequence, pUR2973 contains the α -mating factor signal sequence (see reference 18). Moreover the plasmid pUR2973 contains the *Leu2* marker gene with the complete promoter sequence,
5 instead of the truncated promoter version of pUR2972.

EXAMPLE 4 Immobilized *Geotrichum* lipase/ α -agglutinin on the surface of *S. cerevisiae*

The construction and isolation of the 1.4 kb *NheI/HindIII* fragment comprising the
10 C-terminal part of AG α -1 (α -agglutinin) gene has been described in EXAMPLE 1. For the in-frame gene fusion of the DNA coding for the C-terminal membrane anchor of α -agglutinin to the complete coding sequence of *Geotrichum candidum* lipase B from strain CMICC 335426 (see Figure 8 and SEQ ID NO: 11 and 12), the plasmid pUR2974 can be used. This plasmid, derived from the commercially available
15 pBluescript II SK plasmid, contains the cDNA coding for the complete *G. candidum* lipase II on an 1850 bp long *EcoRI/XhoI* insert (see Figure 9).

To develop an expression vector for *S. cerevisiae* with homologous signal sequences, the N-terminus of the mature lipase B was determined experimentally by standard techniques. The obtained amino acid sequence of "Gln-Ala-Pro-Thr-Ala-Val..." is in
20 complete agreement with the cleavage site of the signal peptidase on the *G. candidum* lipase II (see reference 19).

For the fusion of the mature lipase B to the *S. cerevisiae* signal sequences of *SUC2* (invertase) or α -mating factor (prepro- α MF) on one hand and the in-frame fusion to the 3' part of the AG α 1 gene PCR technique can be used. The PCR primer lipo3
25 (see SEQ ID NO: 13) can be constructed in such a way, that the originally present *EagI* site in the 5'-part of the coding sequence (spanning codons 5-7 of the mature protein) will become inactivated without any alteration in the amino acid sequence. To facilitate the subsequent cloning procedures, the PCR primer can further contain a new *EagI* site at the 5' end, for the in-frame ligation to *SUC2* signal sequence or
30 prepro- α MF sequence, respectively. The corresponding PCR primer lipo4 (see SEQ ID NO: 16) contains an extra *NheI* site behind the nucleotides coding for the

C-terminus of lipase B, to ensure the proper fusion to the C-terminal part of α -agglutinin.

5 PCR oligonucleotides for the in frame linkage of *G. candidum* lipase II to the *SUC2* signal sequence and the C-terminal part of α -agglutinin.

a: N-terminal transition to either prepro α MF sequence or *SUC2* signal sequence.

10 primer lipo3: 5'-GGG ^{EagI} GCG GCC ^A GCG ^Q CAG ^A GCC ^P CCA ^R AGG ^P CGG ^S TCT ^L CTC ^N AAT-3'
lipaseII: 3'-GAC CGG GTC CGG GGT GCC GCC AGA GAG TTA-5'
(non-cod. strand, see SEQ ID NO: 14)

15 b: C-terminal fusion to C part of α -agglutinin

20 lipase: 5'-CA ^S AAC ^N TTT ^F GAG ^E ACT ^T GAC ^D GTT ^V AAT ^N CTC ^L TAC ^Y GGT ^G TAA AAC-3'
(cod. strand)
primer lipo4: 3'-C TGA CTG CAA TTA GAG ATG CCA CGATCG CCCC-5'
(for the part of the lipase coding strand see SEQ ID NO: 15)

The PCR product with the modified ends can be generated by standard PCR
25 protocols, using instead of the normal *Ampli-Taq* polymerase the new thermostable VENT polymerase, which also exhibits proofreading activity, to ensure an error-free DNA template. Through digestion of the formerly described plasmid pUR2972 with *EagI* (complete) and *NheI* (partial), the *Humicola* lipase fragment can be exchanged against the DNA fragment coding for lipase B, thereby generating the final *S. cerevisiae* expression vector pUR2975 (see Figure 9).

The *Humicola* lipase- α -agglutinin fusion protein coding sequence can be exchanged against the lipase B/ α -agglutinin fusion construct described above by digestion of the described vector pUR2973 with *EagI*/*HindIII*, resulting in pUR2976 (see Figure 9).

35 **EXAMPLE 5** Immobilized *Rhizomucor miehei* lipase/ α -agglutinin on the surface of *S. cerevisiae*

The construction and isolation of the 1.4 kb *NheI*/*HindIII* fragment encoding the C-terminal part of α -agglutinin has been described in EXAMPLE 1. The plasmid pUR2980 contains a 1.25 kb cDNA fragment cloned into the *SmaI* site of
40 commercially available pUC18, which (synthetically synthesizable) fragment encodes

the complete coding sequence of triglyceride lipase of *Rhizomucor miehei* (see reference 20), an enzyme used in a number of processes to interesterify triacylglycerols (see reference 21) or to prepare biosurfactants (see reference 22). Beside the 269 codons of the mature lipase molecule, the fragment also harbours

5 codons for the 24 amino acid signal peptide as well as 70 amino acids of the propeptide. PCR can easily be applied to ensure the proper fusion of the gene fragment encoding the mature lipase to the *SUC2* signal sequence or the prepro α -mating factor sequence of *S. cerevisiae*, as well as the in-frame fusion to the described *NheI*/*HindIII* fragment. The following two primers, lipo5 (see SEQ ID NO: 17) and

10 lipo6 (see SEQ ID NO: 20), will generate a 833 bp DNA fragment, which after Proteinase K treatment and digestion with *EagI* and *NheI* can be cloned as an 816 bp long fragment into the *EagI*/*NheI* digested plasmids pUR2972 and pUR2973, respectively (see Figure 7).

15 lipo5: 5'-CCC GCG GCC GCG ^{EagI} ^A ^S ^I ^D ^G ^G ^I AGC ATT GAT GGT GGT ATC-3'
 lipase (non-cod. strand): 3'-TCG TAA CTA GCA CCA TAG-5'
 (for the part of the lipase non-coding strand see SEQ ID NO: 18)

20 lipase (cod. strand): 5'-AAC ACA GGC CTC TGT ACT-3'
 Lipo6: 3'-TTG TGT CCG GAG ACA TGA CGATCGCGCC-5'
 (for the part of the lipase coding strand see SEQ ID NO: 19)

These new *S. cerevisiae* expression plasmids contain the *GAL7* promoter, the invertase signal sequence (pUR2981) or the prepro- α -mating factor sequence (pUR2982), the chimeric *Rhizomucor miehei* lipase/ α -agglutinin gene, the 2 μ m sequence, the

30 defective (truncated) *Leu2* promoter and the *Leu2* gene. These plasmids can be transformed into *S. cerevisiae* and grown and analyzed using protocols described in earlier EXAMPLES.

35 EXAMPLE 6 Immobilized *Aspergillus niger* glucose oxidase/GPI anchored cell wall proteins on the surface of *S. cerevisiae*

Glucose oxidase (β -D:oxygen 1-oxidoreductase, EC 1.1.3.4) from *Aspergillus niger* catalyses the oxidation of β -D-glucose to glucono- δ -lactone and the concomitant reduction of molecular oxygen to hydrogen peroxide. The fungal enzyme consists of a homodimer of molecular weight 150,000 containing two tightly bound FAD co-factors.

Beside the use in glucose detection kits the enzyme is useful as a source of hydrogen peroxide in food preservation. The gene was cloned from both cDNA and genomic libraries, the single open reading frame contains no intervening sequences and encodes a protein of 605 amino acids (see reference 23).

- 5 With the help of two proper oligonucleotides the coding part of the sequence is adjusted in a one-step modifying procedure by PCR in such a way that a fusion gene product will be obtained coding for glucose oxidase and the C-terminal cell wall anchor of the *FLO1* gene product or α -agglutinin. Thus, some of the plasmids described in former EXAMPLES can be utilized to integrate the corresponding
10 sequence in-frame between one of the signal sequences used in the EXAMPLES and the *NheI/HindIII* part of the AG α 1 gene.

Since dimerisation of the two monomers might be a prerequisite for activity, in an alternative approach the complete coding sequence for glucose oxidase without the GPI anchor can be expressed in *S. cerevisiae* transformant which already contains the
15 fusion construct. This can be fulfilled by constitutive expression of the fusion construct containing the GPI anchor with the help of the *GAPDH* or *PGK* promoter for example. The unbound not-anchored monomer can be produced by using a DNA construct comprising an inducible promoter, as for instance the *GAL7* promoter.

20

EXAMPLE 7 Process to convert raffinose, stachyose and similar sugars in soy extracts with α -galactosidase/ α -agglutinin immobilized on yeasts

The yeast transformed with plasmid pUR2969 can be cultivated on large scale. At regular intervals during cultivation the washed cells should be analyzed on the
25 presence of α -galactosidase activity on their surface with methods described in EXAMPLE 1. When both cell density and α -galactosidase activity/biomass reach their maximum, the yeast cells can then be collected by centrifugation and washed. The washed cells can then be added to soy extracts. The final concentration of the yeast cells can vary between 0.1 and 10 g/l, preferably the concentration should be
30 above 1 g/l. The temperature of the soy extract should be $< 8^{\circ}\text{C}$ to reduce the metabolic activity of the yeast cells. The conversion of raffinose and stachyose can be analyzed with HPLC methods and after 95 % conversion of these sugars the yeasts

cells can be removed by centrifugation and their α -galactosidase activity/g biomass can be measured. Centrifugates with a good activity can be used in a subsequent conversion process, whereas centrifugates with an activity of less than 50 % of the original activity can be resuscitated in the growth medium and the cells can be
5 allowed to recover for 2 to 4 hours. Thereafter the cells can be centrifuged, washed and subsequently be used in a subsequent conversion process.

**EXAMPLE 8 Production of biosurfactants using *Humicola*
lipase/ α -agglutinin immobilized on yeasts.**

10 The yeast transformed with plasmid pUR2972 or pUR2973 can be cultivated on large scale. At regular intervals during cultivation the washed cells can be analyzed on the presence of lipase activity on their surface with methods described in EXAMPLE 1. When both cell density and lipase/biomass reach their maximum, the yeast cells can be collected by centrifugation and washed. The washed cells can be suspended in a
15 small amount of water and added to a reactor tank containing a mix of fatty acids, preferably of a chain length between 12-18 carbon atoms and sugars, preferably glucose, galactose or sucrose. The total concentration of the water (excluding the water in the yeast cells) might be below 0.1 %. The final concentration of the yeast cells can vary between 0.1 and 10 g/l, preferably the concentration is above 1 g/l. The
20 tank has to be kept under an atmosphere of N_2 and CO_2 in order to avoid oxidation of the (unsaturated) fatty acids and to minimize the metabolic activity of the yeasts. The temperature of mixture in the tank should be between 30-60 °C, depending on type of fatty acid used. The conversion of fatty acids can be analyzed with GLC methods and after 95 % conversion of these fatty acids the yeasts cells can be
25 removed by centrifugation and their lipase activity/g biomass can be measured. Centrifugates with a good activity can be used in a subsequent conversion process, whereas centrifugates with an activity of less than 50 % of the original activity can be resuscitated in the growth medium and the cells can be allowed to recover for 2 to 8 hours. Thereafter the cells can be centrifuged again, washed and used in a subsequent
30 conversion process.

EXAMPLE 9 Production of special types of triacylglycerols using *Rhizomucor miehei* lipase/ α -agglutinin immobilized on yeasts.

The yeast transformed with plasmid pUR2981 or pUR2982 can be cultivated on a large scale. At regular intervals during cultivation the washed cells can be analyzed on the presence of lipase activity on their surface with methods described in EXAMPLE 1. When both cell density and lipase/biomass reach their maximum, the yeast cells can be collected by centrifugation and washed. The washed cells can be suspended in a small amount of water and can be added to a reactor tank containing a mix of various triacylglycerols and fatty acids. The total concentration of the water (excluding the water in the yeast cells) might be below 0.1 %. The final concentration of the yeast cells can vary between 0.1 and 10 g/l, preferably the concentration is above 1 g/l. The tank has to be kept under an atmosphere of N₂ and CO₂ in order to avoid oxidation of the (unsaturated) fatty acids and to minimize the metabolic activity of the yeasts. The temperature of mixture in the tank should be between 30-70 °C, depending on types of triacylglycerol and fatty acid used. The degree of interesterification can be analyzed with GLC/MS methods and after formation of at least 80 % of the theoretical value of the desired type of triacylglycerol the yeasts cells can be removed by centrifugation and their lipase activity/g biomass can be measured. Centrifugates with a good activity can be used in a subsequent conversion process, whereas centrifugates with an activity of less then 50 % of the original activity is resuscitated in the growth medium and the cells should be allowed to recover 2 to 8 hours. After that the cells can be centrifuged, washed and used in a subsequent inter-esterification process.

Baker's yeasts of strain MT302/1C, transformed with either plasmid pSY13 or plasmid pUR2969 (described in EXAMPLE 1) were deposited under the Budapest Treaty at the Centraalbureau voor Schimmelcultures (CBS) on 3 July 1992 under provisional numbers 330.92 and 329.92, respectively.

EXAMPLE 10 Immobilized *Humicola* lipase/FLO1 fusion on the surface of *S. cerevisiae*

Flocculation, defined as "the (reversible) aggregation of dispersed yeast cells into flocs" (see reference 24), is the most important feature of yeast strains in industrial

fermentations. Beside this it is of principal interest, because it is a property associated with cell wall proteins and it is a quantitative characteristic. One of the genes associated with the flocculation phenotype in *S. cerevisiae* is the *FLO1* gene. The gene is located at approximately 24 kb from the right end of chromosome I and the DNA
5 sequence of a clone containing major parts of *FLO1* gene has very recently been determined (see reference 26). The sequence is given in Figure 11 and SEQ ID NO: 21 and 22. The cloned fragment appeared to be approximately 2 kb shorter than the genomic copy as judged from Southern and Northern hybridizations, but encloses both ends of the *FLO1* gene. Analysis of the DNA sequence data indicates that the
10 putative protein contains at the N-terminus a hydrophobic region which confirms a signal sequence for secretion, a hydrophobic C-terminus that might function as a signal for the attachment of a GPI-anchor and many glycosylation sites, especially in the C-terminus, with 46,6 % serine and threonine in the arbitrarily defined C-terminus (aa 271-894). Hence, it is likely that the *FLO1* gene product is localized in an
15 orientated fashion in the yeast cell wall and may be directly involved in the process of interaction with neighbouring cells. The cloned *FLO1* sequence might therefore be suitable for the immobilization of proteins or peptides on the cell surface by a different type of cell wall anchor.

Recombinant DNA constructs can be obtained, for example by utilizing the DNA
20 coding for amino acids 271-894 of the *FLO1* gene product, i.e. polynucleotide 811-2682 of Figure 11. Through application of two PCR primers pcrflo1 (see SEQ ID NO: 23) and pcrflo2 (see SEQ ID NO: 26) *NheI* and *HindIII* sites can be introduced at both ends of the DNA fragment. In a second step, the 1.4 kb *NheI/HindIII* fragment present in pUR2972 (either A or B) containing the C-terminal part of
25 α -agglutinin can be replaced by the 1.9 kb DNA fragment coding for the C-terminal part of the *FLO1* protein, resulting in plasmid pUR2990 (see Figure 12), comprising a DNA sequence encoding (a) the invertase signal sequence (*SUC2*) preceding (b) the fusion protein consisting of (b.1) the lipase of *Humicola* (see reference 16) followed by (b.2) the C-terminus of *FLO1* protein (aa 271-894).

Plasmid pUR2972 (either A or B) can be restricted with *Nhe*I (partial) and *Hind*III and the *Nhe*I/*Hind*III fragment comprising the vector backbone and the lipase gene can be ligated to the correspondingly digested PCR product of the plasmid containing the *FLO1* sequence, resulting in plasmid pUR2990, containing the *GAL7* promoter, the *S. cerevisiae* invertase signal sequence, the chimeric lipase/*FLO1* gene, the yeast 2 μ m sequence, the defective *Leu2* promoter and the *Leu2* gene. This plasmid can be transformed into *S. cerevisiae* and the transformed cells can be cultivated in YP medium including galactose as inductor.

The expression, secretion, localization and activity of the chimeric lipase/*FLO1* protein can be analyzed using similar procedures as given in Example 1.

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30 by *Saccharomyces cerevisiae* Gene 125 115-123

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- (ii) TITLE OF INVENTION: Enzymic Processes based on naturally immobilized enzymes that can easily be separated and regenerated

(iii) NUMBER OF SEQUENCES: 26

(iv) COMPUTER READABLE FORM:

- (A) MEDIUM TYPE: Floppy disk
- (B) COMPUTER: IBM PC compatible
- (C) OPERATING SYSTEM: PC-DOS/MS-DOS
- (D) SOFTWARE: PatentIn Release #1.0, Version #1.25 (EPO)

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 6057 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(vi) ORIGINAL SOURCE:

- (A) ORGANISM: *Saccharomyces cerevisiae*

(ix) FEATURE:

- (A) NAME/KEY: CDS
- (B) LOCATION: 3653..5605
- (D) OTHER INFORMATION: /function= "sexual agglutination"
/product= "alpha-agglutinin"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

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Val	Ile	Ser	His	Val	Val	Thr	Thr	Ser	Thr	Lys	Leu	Ser	Pro	Thr	Ala	
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 TCTTATCCTT TTCCGTCTCA CCGCAGATTT TATCATAGTA TTAAATTTAT ATTTTGTTCG 5802
 TAAAAGAAA AATTTGTGAG CGTTACCGCT CGTTTCATTA CCCGAAGGCT GTTTCAGTAG 5862
 ACCACTGATT AAGTAAGTAG ATGAAAAAAT TTCATCACCA TGAAAGAGTT CGATGAGAGC 5922
 TACTTTTTCA AATGCTTAAC AGCTAACCGC CATTCAATAA TGTTACGTTT TCTTCATTCT 5982
 GCGGCTACGT TATCTAACAA GAGGTTTAC TCTCTCATAT CTCATTCAAA TAGAAAGAAC 6042
 ATAATCAAAA AGCTT 6057

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 650 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

Met Phe Thr Phe Leu Lys Ile Ile Leu Trp Leu Phe Ser Leu Ala Leu
 1 5 10 15
 Ala Ser Ala Ile Asn Ile Asn Asp Ile Thr Phe Ser Asn Leu Glu Ile
 20 25 30
 Thr Pro Leu Thr Ala Asn Lys Gln Pro Asp Gln Gly Trp Thr Ala Thr
 35 40 45
 Phe Asp Phe Ser Ile Ala Asp Ala Ser Ser Ile Arg Glu Gly Asp Glu
 50 55 60

Phe Thr Leu Ser Met Pro His Val Tyr Arg Ile Lys Leu Leu Asn Ser
65 70 75 80

Ser Gln Thr Ala Thr Ile Ser Leu Ala Asp Gly Thr Glu Ala Phe Lys
85 90 95

Cys Tyr Val Ser Gln Gln Ala Ala Tyr Leu Tyr Glu Asn Thr Thr Phe
100 105 110

Thr Cys Thr Ala Gln Asn Asp Leu Ser Ser Tyr Asn Thr Ile Asp Gly
115 120 125

Ser Ile Thr Phe Ser Leu Asn Phe Ser Asp Gly Gly Ser Ser Tyr Glu
130 135 140

Tyr Glu Leu Glu Asn Ala Lys Phe Phe Lys Ser Gly Pro Met Leu Val
145 150 155 160

Lys Leu Gly Asn Gln Met Ser Asp Val Val Asn Phe Asp Pro Ala Ala
165 170 175

Phe Thr Glu Asn Val Phe His Ser Gly Arg Ser Thr Gly Tyr Gly Ser
180 185 190

Phe Glu Ser Tyr His Leu Gly Met Tyr Cys Pro Asn Gly Tyr Phe Leu
195 200 205

Gly Gly Thr Glu Lys Ile Asp Tyr Asp Ser Ser Asn Asn Asn Val Asp
210 215 220

Leu Asp Cys Ser Ser Val Gln Val Tyr Ser Ser Asn Asp Phe Asn Asp
225 230 235 240

Trp Trp Phe Pro Gln Ser Tyr Asn Asp Thr Asn Ala Asp Val Thr Cys
245 250 255

Phe Gly Ser Asn Leu Trp Ile Thr Leu Asp Glu Lys Leu Tyr Asp Gly
260 265 270

Glu Met Leu Trp Val Asn Ala Leu Gln Ser Leu Pro Ala Asn Val Asn
275 280 285

Thr Ile Asp His Ala Leu Glu Phe Gln Tyr Thr Cys Leu Asp Thr Ile
290 295 300

Ala Asn Thr Thr Tyr Ala Thr Gln Phe Ser Thr Thr Arg Glu Phe Ile
305 310 315 320

Val Tyr Gln Gly Arg Asn Leu Gly Thr Ala Ser Ala Lys Ser Ser Phe
325 330 335

Ile Ser Thr Thr Thr Thr Asp Leu Thr Ser Ile Asn Thr Ser Ala Tyr
340 345 350

Ser Thr Gly Ser Ile Ser Thr Val Glu Thr Gly Asn Arg Thr Thr Ser
355 360 365

Glu Val Ile Ser His Val Val Thr Thr Ser Thr Lys Leu Ser Pro Thr
370 375 380

Ala Thr Thr Ser Leu Thr Ile Ala Gln Thr Ser Ile Tyr Ser Thr Asp
385 390 395 400

Ser Asn Ile Thr Val Gly Thr Asp Ile His Thr Thr Ser Glu Val Ile
405 410 415

Ser Asp Val Glu Thr Ile Ser Arg Glu Thr Ala Ser Thr Val Val Ala
420 425 430

Ala Pro Thr Ser Thr Thr Gly Trp Thr Gly Ala Met Asn Thr Tyr Ile
435 440 445

Pro Gln Phe Thr Ser Ser Ser Phe Ala Thr Ile Asn Ser Thr Pro Ile
450 455 460

Ile Ser Ser Ser Ala Val Phe Glu Thr Ser Asp Ala Ser Ile Val Asn
465 470 475 480

Val His Thr Glu Asn Ile Thr Asn Thr Ala Ala Val Pro Ser Glu Glu
485 490 495

Pro Thr Phe Val Asn Ala Thr Arg Asn Ser Leu Asn Ser Phe Cys Ser
500 505 510

Ser Lys Gln Pro Ser Ser Pro Ser Ser Tyr Thr Ser Ser Pro Leu Val
515 520 525

Ser Ser Leu Ser Val Ser Lys Thr Leu Leu Ser Thr Ser Phe Thr Pro
530 535 540

42

Ser Val Pro Thr Ser Asn Thr Tyr Ile Lys Thr Glu Asn Thr Gly Tyr
 545 550 555 560

Phe Glu His Thr Ala Leu Thr Thr Ser Ser Val Gly Leu Asn Ser Phe
 565 570 575

Ser Glu Thr Ala Leu Ser Ser Gln Gly Thr Lys Ile Asp Thr Phe Leu
 580 585 590

Val Ser Ser Leu Ile Ala Tyr Pro Ser Ser Ala Ser Gly Ser Gln Leu
 595 600 605

Ser Gly Ile Gln Gln Asn Phe Thr Ser Thr Ser Leu Met Ile Ser Thr
 610 615 620

Tyr Glu Gly Lys Ala Ser Ile Phe Phe Ser Ala Glu Leu Gly Ser Ile
 625 630 635 640

Ile Phe Leu Leu Leu Ser Tyr Leu Leu Phe
 645 650

(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 29 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(vii) IMMEDIATE SOURCE:

- (B) CLONE: primer lipol

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

GGGGCGGCCG AGGTCTCGCA AGATCTGGA

(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 33 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(vii) IMMEDIATE SOURCE:

- (B) CLONE: Part non-coding strand lipase

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

TTGTGCCAGG TCTTGGGAGA CCTCTGACG AAT

33

(2) INFORMATION FOR SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 32 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(vii) IMMEDIATE SOURCE:

- (B) CLONE: Part coding strand lipase

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

TTCGGGTTAA TTGGGACATG TCTTTAGTGC GA

32

(2) INFORMATION FOR SEQ ID NO: 6:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 40 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

44

(vii) IMMEDIATE SOURCE:

(B) CLONE: primer lipo2

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

CCCCAAGCTT AAGGCTAGCA AGACATGTCC CAATTAACCC

40

(2) INFORMATION FOR SEQ ID NO: 7:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 894 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: double

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(vi) ORIGINAL SOURCE:

(A) ORGANISM: *Humicola lanuginosa*

(ix) FEATURE:

(A) NAME/KEY: CDS

(B) LOCATION: 72..884

(D) OTHER INFORMATION: /product= "lipase"

(ix) FEATURE:

(A) NAME/KEY: mat_peptide

(B) LOCATION: 72..881

(D) OTHER INFORMATION: /product= "lipase"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

GAATTCGTAG CGACGATATG AGGAGCTCCC TTGTGCTGTT CTTGTCTCT GCGTGGACGG 60

CCTTGCCAC G GCC GAG GTC TCG CAA GAT CTG TTT AAC CAG TTC AAT CTC 110

Ala Glu Val Ser Gln Asp Leu Phe Asn Gln Phe Asn Leu

1

5

10

TTT GCA CAG TAT TCT GCT GCC GCA TAC TGC GGA AAA AAC AAT GAT GCC 158

Phe Ala Gln Tyr Ser Ala Ala Ala Tyr Cys Gly Lys Asn Asn Asp Ala

15

20

25

45

CCA GCT GGT ACA AAC ATT ACG TGC ACG GGA AAT GCC TGC CCC GAG GTA	206
Pro Ala Gly Thr Asn Ile Thr Cys Thr Gly Asn Ala Cys Pro Glu Val	
30 35 40 45	
GAG AAG GCG GAT GCA ACG TTT CTC TAC TCG TTT GAA GAC TCT GGA GTG	254
Glu Lys Ala Asp Ala Thr Phe Leu Tyr Ser Phe Glu Asp Ser Gly Val	
50 55 60	
GGC GAT GTC ACC GGC TTC CTT GCT CTA GAC AAC ACG AAC AAA TTG ATC	302
Gly Asp Val Thr Gly Phe Leu Ala Leu Asp Asn Thr Asn Lys Leu Ile	
65 70 75	
GTC CTC TCT TTC CGT GGC TCT CGT TCC ATA GAA AAC TGG ATC GGA AAT	350
Val Leu Ser Phe Arg Gly Ser Arg Ser Ile Glu Asn Trp Ile Gly Asn	
80 85 90	
CTT AAC TTC GAC TTG AAA GAA ATA AAT GAC ATT TGC TCC GGC TGC AGG	398
Leu Asn Phe Asp Leu Lys Glu Ile Asn Asp Ile Cys Ser Gly Cys Arg	
95 100 105	
GGA CAT GAC GGC TTC ACC TCG AGC TGG AGG TCT GTA GCC GAT ACG TTA	446
Gly His Asp Gly Phe Thr Ser Ser Trp Arg Ser Val Ala Asp Thr Leu	
110 115 120 125	
AGG CAG AAG GTG GAG GAT GCT GTG AGG GAG CAT CCC GAC TAT CGC GTG	494
Arg Gln Lys Val Glu Asp Ala Val Arg Glu His Pro Asp Tyr Arg Val	
130 135 140	
GTG TTT ACC GGA CAT AGC TTG GGT GGT GCA TTG GCA ACT GTT GCC GGA	542
Val Phe Thr Gly His Ser Leu Gly Gly Ala Leu Ala Thr Val Ala Gly	
145 150 155	
GCA GAC CTG CGT GGA AAT GGG TAT GAC ATC GAC GTG TTT TCA TAT GGC	590
Ala Asp Leu Arg Gly Asn Gly Tyr Asp Ile Asp Val Phe Ser Tyr Gly	
160 165 170	
GCC CCC CGA GTC GGA AAC AGG GCT TTT GCA GAA TTC CTG ACC GTA CAG	638
Ala Pro Arg Val Gly Asn Arg Ala Phe Ala Glu Phe Leu Thr Val Gln	
175 180 185	
ACC GGC GGT ACC CTC TAC CGC ATT ACC CAC ACC AAT GAT ATT GTC CCT	686
Thr Gly Gly Thr Leu Tyr Arg Ile Thr His Thr Asn Asp Ile Val Pro	
190 195 200 205	

46

AGA CTC CCG CCG CGC GAG TTC GGT TAC AGC CAT TCT AGC CCA GAG TAC	734
Arg Leu Pro Pro Arg Glu Phe Gly Tyr Ser His Ser Ser Pro Glu Tyr	
210 215 220	
TGG ATC AAA TCT GGA ACC CTT GTC CCC GTC ACC CGA AAC GAC ATC GTG	782
Trp Ile Lys Ser Gly Thr Leu Val Pro Val Thr Arg Asn Asp Ile Val	
225 230 235	
AAG ATA GAA GGC ATC GAT GCC ACC GGC GGC AAT AAC CAG CCT AAC ATT	830
Lys Ile Glu Gly Ile Asp Ala Thr Gly Gly Asn Asn Gln Pro Asn Ile	
240 245 250	
CCG GAT ATC CCT GCG CAC CTA TGG TAC TTC GGG TTA ATT GGG ACA TGT	878
Pro Asp Ile Pro Ala His Leu Trp Tyr Phe Gly Leu Ile Gly Thr Cys	
255 260 265	
CTT TAGTGCGAAG CTT	894
Leu	
270	

(2) INFORMATION FOR SEQ ID NO: 8:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 270 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

Ala Glu Val Ser Gln Asp Leu Phe Asn Gln Phe Asn Leu Phe Ala Gln
1 5 10 15
Tyr Ser Ala Ala Ala Tyr Cys Gly Lys Asn Asn Asp Ala Pro Ala Gly
20 25 30
Thr Asn Ile Thr Cys Thr Gly Asn Ala Cys Pro Glu Val Glu Lys Ala
35 40 45
Asp Ala Thr Phe Leu Tyr Ser Phe Glu Asp Ser Gly Val Gly Asp Val
50 55 60

Thr Gly Phe Leu Ala Leu Asp Asn Thr Asn Lys Leu Ile Val Leu Ser
 65 70 75 80

Phe Arg Gly Ser Arg Ser Ile Glu Asn Trp Ile Gly Asn Leu Asn Phe
 85 90 95

Asp Leu Lys Glu Ile Asn Asp Ile Cys Ser Gly Cys Arg Gly His Asp
 100 105 110

Gly Phe Thr Ser Ser Trp Arg Ser Val Ala Asp Thr Leu Arg Gln Lys
 115 120 125

Val Glu Asp Ala Val Arg Glu His Pro Asp Tyr Arg Val Val Phe Thr
 130 135 140

Gly His Ser Leu Gly Gly Ala Leu Ala Thr Val Ala Gly Ala Asp Leu
 145 150 155 160

Arg Gly Asn Gly Tyr Asp Ile Asp Val Phe Ser Tyr Gly Ala Pro Arg
 165 170 175

Val Gly Asn Arg Ala Phe Ala Glu Phe Leu Thr Val Gln Thr Gly Gly
 180 185 190

Thr Leu Tyr Arg Ile Thr His Thr Asn Asp Ile Val Pro Arg Leu Pro
 195 200 205

Pro Arg Glu Phe Gly Tyr Ser His Ser Ser Pro Glu Tyr Trp Ile Lys
 210 215 220

Ser Gly Thr Leu Val Pro Val Thr Arg Asn Asp Ile Val Lys Ile Glu
 225 230 235 240

Gly Ile Asp Ala Thr Gly Gly Asn Asn Gln Pro Asn Ile Pro Asp Ile
 245 250 255

Pro Ala His Leu Trp Tyr Phe Gly Leu Ile Gly Thr Cys Leu
 260 265 270

(2) INFORMATION FOR SEQ ID NO: 9:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 55 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(vii) IMMEDIATE SOURCE:

- (B) CLONE: primer

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:

ATCCCTGCGC ACCTATGGTA CTTCGGGTTA ATTGGGACAT GTCTTGCTAG CCTTA

55

(2) INFORMATION FOR SEQ ID NO: 10:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 59 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(vii) IMMEDIATE SOURCE:

- (B) CLONE: primer

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 10:

AGCTTAAGGC TAGCAAGACA TGTCCCAATT AACCCGAAGT ACCATAGGTG CGCAGGGAT

59

(2) INFORMATION FOR SEQ ID NO: 11:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1828 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA to mRNA

(vi) ORIGINAL SOURCE:

- (A) ORGANISM: *Geotrichum candidum*
 (B) STRAIN: CMICC 335426

(ix) FEATURE:

- (A) NAME/KEY: CDS
 (B) LOCATION: 40..1731
 (D) OTHER INFORMATION: /product= "lipase"

(ix) FEATURE:

- (A) NAME/KEY: sig_peptide
 (B) LOCATION: 40..96

(ix) FEATURE:

- (A) NAME/KEY: mat_peptide
 (B) LOCATION: 97..1728
 (D) OTHER INFORMATION: /product= "lipase"
 /gene= "lipB"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 11:

AATTCGGCAC GAGATTCCTT TGATTGCAA CTGTTAATC ATG GTT TCC AAA AGC	54
Met Val Ser Lys Ser	
-19 -15	
TTT TTT TTG GCT GCG GCG CTC AAC GTA GTG GGC ACC TTG GCC CAG GCC	102
Phe Phe Leu Ala Ala Ala Leu Asn Val Val Gly Thr Leu Ala Gln Ala	
-10 -5 1	
CCC ACG GCC GTT CTT AAT GGC AAC GAG GTC ATC TCT GGT GTC CTT GAG	150
Pro Thr Ala Val Leu Asn Gly Asn Glu Val Ile Ser Gly Val Leu Glu	
5 10 15	
GGC AAG GTT GAT ACC TTC AAG GGA ATC CCA TTT GCT GAC CCT CCT GTT	198
Gly Lys Val Asp Thr Phe Lys Gly Ile Pro Phe Ala Asp Pro Pro Val	
20 25 30	
GGT GAC TTG CGG TTC AAG CAC CCC CAG CCT TTC ACT GGA TCC TAC CAG	246
Gly Asp Leu Arg Phe Lys His Pro Gln Pro Phe Thr Gly Ser Tyr Gln	
35 40 45 50	
GGT CTT AAG GCC AAC GAC TTC AGC TCT GCT TGT ATG CAG CTT GAT CCT	294
Gly Leu Lys Ala Asn Asp Phe Ser Ser Ala Cys Met Gln Leu Asp Pro	
55 60 65	

50

GGC AAT GCC TTT TCT TTG CTT GAC AAA GTA GTG GGC TTG GGA AAG ATT	342
Gly Asn Ala Phe Ser Leu Leu Asp Lys Val Val Gly Leu Gly Lys Ile	
70 75 80	
CTT CCT GAT AAC CTT AGA GGC CCT CTT TAT GAC ATG GCC CAG GGT AGT	390
Leu Pro Asp Asn Leu Arg Gly Pro Leu Tyr Asp Met Ala Gln Gly Ser	
85 90 95	
GTC TCC ATG AAT GAG GAC TGT CTC TAC CTT AAC GTT TTC CGC CCC GCT	438
Val Ser Met Asn Glu Asp Cys Leu Tyr Leu Asn Val Phe Arg Pro Ala	
100 105 110	
GGC ACC AAG CCT GAT GCT AAG CTC CCC GTC ATG GTT TGG ATT TAC GGT	486
Gly Thr Lys Pro Asp Ala Lys Leu Pro Val Met Val Trp Ile Tyr Gly	
115 120 125 130	
GGT GCC TTT GTG TTT GGT TCT TCT GCT TCT TAC CCT GGT AAC GGC TAC	534
Gly Ala Phe Val Phe Gly Ser Ser Ala Ser Tyr Pro Gly Asn Gly Tyr	
135 140 145	
GTC AAG GAG AGT GTG GAA ATG GGC CAG CCT GTT GTG TTT GTT TCC ATC	582
Val Lys Glu Ser Val Glu Met Gly Gln Pro Val Val Phe Val Ser Ile	
150 155 160	
AAC TAC CGT ACC GGC CCC TAT GGA TTC TTG GGT GGT GAT GCC ATC ACC	630
Asn Tyr Arg Thr Gly Pro Tyr Gly Phe Leu Gly Gly Asp Ala Ile Thr	
165 170 175	
GCT GAG GGC AAC ACC AAC GCT GGT CTG CAC GAC CAG CGC AAG GGT CTC	678
Ala Glu Gly Asn Thr Asn Ala Gly Leu His Asp Gln Arg Lys Gly Leu	
180 185 190	
GAG TGG GTT AGC GAC AAC ATT GCC AAC TTT GGT GGT GAT CCC GAC AAG	726
Glu Trp Val Ser Asp Asn Ile Ala Asn Phe Gly Gly Asp Pro Asp Lys	
195 200 205 210	
GTC ATG ATT TTC GGT GAG TCC GCT GGT GCC ATG AGT GTT GCT CAC CAG	774
Val Met Ile Phe Gly Glu Ser Ala Gly Ala Met Ser Val Ala His Gln	
215 220 225	
CTT GTT GCC TAC GGT GGT GAC AAC ACC TAC AAC GGA AAG CAG CTT TTC	822
Leu Val Ala Tyr Gly Gly Asp Asn Thr Tyr Asn Gly Lys Gln Leu Phe	
230 235 240	

CAC TCT GCC ATT CTT CAG TCT GGC GGT CCT CTT CCT TAC TTT GAC TCT	870
His Ser Ala Ile Leu Gln Ser Gly Gly Pro Leu Pro Tyr Phe Asp Ser	
245 250 255	
ACT TCT GTT GGT CCC GAG AGT GCC TAC AGC AGA TTT GCT CAG TAT GCC	918
Thr Ser Val Gly Pro Glu Ser Ala Tyr Ser Arg Phe Ala Gln Tyr Ala	
260 265 270	
GGA TGT GAC ACC AGT GCC AGT GAT AAT GAC ACT CTG GCT TGT CTC CGC	966
Gly Cys Asp Thr Ser Ala Ser Asp Asn Asp Thr Leu Ala Cys Leu Arg	
275 280 285 290	
AGC AAG TCC AGC GAT GTC TTG CAC AGT GCG CAG AAC TCG TAT GAT CTT	1014
Ser Lys Ser Ser Asp Val Leu His Ser Ala Gln Asn Ser Tyr Asp Leu	
295 300 305	
AAG GAC CTG TTT GGT CTG CTC CCT CAA TTC CTT GGA TTT GGT CCC AGA	1062
Lys Asp Leu Phe Gly Leu Leu Pro Gln Phe Leu Gly Phe Gly Pro Arg	
310 315 320	
CCC GAC GGC AAC ATT ATT CCC GAT GCC GCT TAT GAG CTC TAC CGC AGC	1110
Pro Asp Gly Asn Ile Ile Pro Asp Ala Ala Tyr Glu Leu Tyr Arg Ser	
325 330 335	
GGT AGA TAC GCC AAG GTT CCC TAC ATT ACT GGC AAC CAG GAG GAT GAG	1158
Gly Arg Tyr Ala Lys Val Pro Tyr Ile Thr Gly Asn Gln Glu Asp Glu	
340 345 350	
GGT ACT ATT CTT GCG CCC GTT GCT ATT AAT GCT ACC ACT ACT CCC CAT	1206
Gly Thr Ile Leu Ala Pro Val Ala Ile Asn Ala Thr Thr Thr Pro His	
355 360 365 370	
GTT AAG AAG TGG TTG AAG TAC ATT TGT AGC CAG GCT TCT GAC GCT TCG	1254
Val Lys Lys Trp Leu Lys Tyr Ile Cys Ser Gln Ala Ser Asp Ala Ser	
375 380 385	
CTT GAT CGT GTT TTG TCG CTC TAC CCC GGC TCT TGG TCG GAG GGT TCA	1302
Leu Asp Arg Val Leu Ser Leu Tyr Pro Gly Ser Trp Ser Glu Gly Ser	
390 395 400	
CCA TTC CGC ACT GGT ATT CTT AAT GCT CTT ACC CCT CAG TTC AAG CGC	1350
Pro Phe Arg Thr Gly Ile Leu Asn Ala Leu Thr Pro Gln Phe Lys Arg	
405 410 415	

52

ATT GCT GCC ATT TTC ACT GAT TTG CTG TTC CAG TCT CCT CGT CGT GTT Ile Ala Ala Ile Phe Thr Asp Leu Leu Phe Gln Ser Pro Arg Arg Val 420 425 430	1398
ATG CTT AAC GCT ACC AAG GAC GTC AAC CGC TGG ACT TAC CTT GCC ACC Met Leu Asn Ala Thr Lys Asp Val Asn Arg Trp Thr Tyr Leu Ala Thr 435 440 445 450	1446
CAG CTC CAT AAC CTC GTT CCA TTT TTG GGT ACT TTC CAT GGC AGT GAT Gln Leu His Asn Leu Val Pro Phe Leu Gly Thr Phe His Gly Ser Asp 455 460 465	1494
CTT CTT TTT CAA TAC TAC GTG GAC CTT GGC CCA TCT TCT GCT TAC CGC Leu Leu Phe Gln Tyr Tyr Val Asp Leu Gly Pro Ser Ser Ala Tyr Arg 470 475 480	1542
CGC TAC TTT ATC TCG TTT GCC AAC CAC CAC GAC CCC AAC GTT GGT ACC Arg Tyr Phe Ile Ser Phe Ala Asn His His Asp Pro Asn Val Gly Thr 485 490 495	1590
AAC CTC CAA CAG TGG GAT ATG TAC ACT GAT GCA GGC AAG GAG ATG CTT Asn Leu Gln Gln Trp Asp Met Tyr Thr Asp Ala Gly Lys Glu Met Leu 500 505 510	1638
CAG ATT CAT ATG ATT GGT AAC TCT ATG AGA ACT GAC GAC TTT AGA ATC Gln Ile His Met Ile Gly Asn Ser Met Arg Thr Asp Asp Phe Arg Ile 515 520 525 530	1686
GAG GGA ATC TCG AAC TTT GAG TCT GAC GTT ACT CTC TTC GGT TAATCCCATT Glu Gly Ile Ser Asn Phe Glu Ser Asp Val Thr Leu Phe Gly 535 540 545	1738
TAGCAAGTTT TGTGTATTTC AAGTATACCA GTTGATGTAA TATATCAATA GATTACAAAT	1798
TAATTAGTGA AAAAAAAAAA AAAAAAAAAAC	1828

(2) INFORMATION FOR SEQ ID NO: 12:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 563 amino acids
 - (B) TYPE: amino acid
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 12:

Met Val Ser Lys Ser Phe Phe Leu Ala Ala Ala Leu Asn Val Val Gly
-19 -15 -10 -5

Thr Leu Ala Gln Ala Pro Thr Ala Val Leu Asn Gly Asn Glu Val Ile
1 5 10

Ser Gly Val Leu Glu Gly Lys Val Asp Thr Phe Lys Gly Ile Pro Phe
15 20 25

Ala Asp Pro Pro Val Gly Asp Leu Arg Phe Lys His Pro Gln Pro Phe
30 35 40 45

Thr Gly Ser Tyr Gln Gly Leu Lys Ala Asn Asp Phe Ser Ser Ala Cys
50 55 60

Met Gln Leu Asp Pro Gly Asn Ala Phe Ser Leu Leu Asp Lys Val Val
65 70 75

Gly Leu Gly Lys Ile Leu Pro Asp Asn Leu Arg Gly Pro Leu Tyr Asp
80 85 90

Met Ala Gln Gly Ser Val Ser Met Asn Glu Asp Cys Leu Tyr Leu Asn
95 100 105

Val Phe Arg Pro Ala Gly Thr Lys Pro Asp Ala Lys Leu Pro Val Met
110 115 120 125

Val Trp Ile Tyr Gly Gly Ala Phe Val Phe Gly Ser Ser Ala Ser Tyr
130 135 140

Pro Gly Asn Gly Tyr Val Lys Glu Ser Val Glu Met Gly Gln Pro Val
145 150 155

Val Phe Val Ser Ile Asn Tyr Arg Thr Gly Pro Tyr Gly Phe Leu Gly
160 165 170

Gly Asp Ala Ile Thr Ala Glu Gly Asn Thr Asn Ala Gly Leu His Asp
175 180 185

Gln Arg Lys Gly Leu Glu Trp Val Ser Asp Asn Ile Ala Asn Phe Gly
190 195 200 205

Gly Asp Pro Asp Lys Val Met Ile Phe Gly Glu Ser Ala Gly Ala Met
 210 215 220

Ser Val Ala His Gln Leu Val Ala Tyr Gly Gly Asp Asn Thr Tyr Asn
 225 230 235

Gly Lys Gln Leu Phe His Ser Ala Ile Leu Gln Ser Gly Gly Pro Leu
 240 245 250

Pro Tyr Phe Asp Ser Thr Ser Val Gly Pro Glu Ser Ala Tyr Ser Arg
 255 260 265

Phe Ala Gln Tyr Ala Gly Cys Asp Thr Ser Ala Ser Asp Asn Asp Thr
 270 275 280 285

Leu Ala Cys Leu Arg Ser Lys Ser Ser Asp Val Leu His Ser Ala Gln
 290 295 300

Asn Ser Tyr Asp Leu Lys Asp Leu Phe Gly Leu Leu Pro Gln Phe Leu
 305 310 315

Gly Phe Gly Pro Arg Pro Asp Gly Asn Ile Ile Pro Asp Ala Ala Tyr
 320 325 330

Glu Leu Tyr Arg Ser Gly Arg Tyr Ala Lys Val Pro Tyr Ile Thr Gly
 335 340 345

Asn Gln Glu Asp Glu Gly Thr Ile Leu Ala Pro Val Ala Ile Asn Ala
 350 355 360 365

Thr Thr Thr Pro His Val Lys Lys Trp Leu Lys Tyr Ile Cys Ser Gln
 370 375 380

Ala Ser Asp Ala Ser Leu Asp Arg Val Leu Ser Leu Tyr Pro Gly Ser
 385 390 395

Trp Ser Glu Gly Ser Pro Phe Arg Thr Gly Ile Leu Asn Ala Leu Thr
 400 405 410

Pro Gln Phe Lys Arg Ile Ala Ala Ile Phe Thr Asp Leu Leu Phe Gln
 415 420 425

Ser Pro Arg Arg Val Met Leu Asn Ala Thr Lys Asp Val Asn Arg Trp
 430 435 440 445

55

Thr Tyr Leu Ala Thr Gln Leu His Asn Leu Val Pro Phe Leu Gly Thr
 450 455 460

Phe His Gly Ser Asp Leu Leu Phe Gln Tyr Tyr Val Asp Leu Gly Pro
 465 470 475

Ser Ser Ala Tyr Arg Arg Tyr Phe Ile Ser Phe Ala Asn His His Asp
 480 485 490

Pro Asn Val Gly Thr Asn Leu Gln Gln Trp Asp Met Tyr Thr Asp Ala
 495 500 505

Gly Lys Glu Met Leu Gln Ile His Met Ile Gly Asn Ser Met Arg Thr
 510 515 520 525

Asp Asp Phe Arg Ile Glu Gly Ile Ser Asn Phe Glu Ser Asp Val Thr
 530 535 540

Leu Phe Gly

(2) INFORMATION FOR SEQ ID NO: 13:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 36 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(vii) IMMEDIATE SOURCE:

- (B) CLONE: primer lipo3

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 13:

GGGGCGGCCG CGCAGGCCCC AAGGCGGTCT CTCAAT

36

(2) INFORMATION FOR SEQ ID NO: 14:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 30 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(vii) IMMEDIATE SOURCE:

- (B) CLONE: Part non-coding strand lipaseII

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 14:

ATTGAGAGAC CGCCGTGGGG CCTGGGCCAG

30

(2) INFORMATION FOR SEQ ID NO: 15:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 38 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(vii) IMMEDIATE SOURCE:

- (B) CLONE: Part coding strand lipaseII

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 15:

CAAACCTTTGA GACTGACGTT AATCTCTACG GTTAAAC

38

(2) INFORMATION FOR SEQ ID NO: 16:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 32 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

57

(vii) IMMEDIATE SOURCE:

(B) CLONE: primer lipo4

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 16:

CCCCGCTAGC ACCGTAGAGA TTAACGTCAG TC

32

(2) INFORMATION FOR SEQ ID NO: 17:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 30 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(vii) IMMEDIATE SOURCE:

(B) CLONE: primer lipo5

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 17:

CCCGCGGCCG CGAGCATTGA TGGTGGTATC

30

(2) INFORMATION FOR SEQ ID NO: 18:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 18 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(vii) IMMEDIATE SOURCE:

(B) CLONE: Part non-coding strand lipase

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 18:

GATACCACGA TCAATGCT

18

(2) INFORMATION FOR SEQ ID NO: 19:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 18 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(vii) IMMEDIATE SOURCE:

- (B) CLONE: Part coding strand lipase

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 19:

AACACAGGCC TCTGTACT

18

(2) INFORMATION FOR SEQ ID NO: 20:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 28 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(vii) IMMEDIATE SOURCE:

- (B) CLONE: primer lipo6

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 20:

CCGCGCTAGC AGTACAGAGG CCTGTGTT

28

(2) INFORMATION FOR SEQ ID NO: 21:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 2685 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(vi) ORIGINAL SOURCE:

(A) ORGANISM: *Saccharomyces cerevisiae*

(vii) IMMEDIATE SOURCE:

(B) CLONE: pYY105

(ix) FEATURE:

(A) NAME/KEY: CDS

(B) LOCATION: 1..2685

(D) OTHER INFORMATION: /product= "Flocculation protein"
/gene= "FLO1"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 21:

ATG ACA ATG CCT CAT CGC TAT ATG TTT TTG GCA GTC TTT ACA CTT CTG	48
Met Thr Met Pro His Arg Tyr Met Phe Leu Ala Val Phe Thr Leu Leu	
1 5 10 15	
GCA CTA ACT AGT GTG GCC TCA GGA GCC ACA GAG GCG TGC TTA CCA GCA	96
Ala Leu Thr Ser Val Ala Ser Gly Ala Thr Glu Ala Cys Leu Pro Ala	
20 25 30	
GGC CAG AGG AAA AGT GGG ATG AAT ATA AAT TTT TAC CAG TAT TCA TTG	144
Gly Gln Arg Lys Ser Gly Met Asn Ile Asn Phe Tyr Gln Tyr Ser Leu	
35 40 45	
AAA GAT TCC TCC ACA TAT TCG AAT GCA GCA TAT ATG GCT TAT GGA TAT	192
Lys Asp Ser Ser Thr Tyr Ser Asn Ala Ala Tyr Met Ala Tyr Gly Tyr	
50 55 60	
GCC TCA AAA ACC AAA CTA GGT TCT GTC GGA GGA CAA ACT GAT ATC TCG	240
Ala Ser Lys Thr Lys Leu Gly Ser Val Gly Gly Gln Thr Asp Ile Ser	
65 70 75 80	
ATT GAT TAT AAT ATT CCC TGT GTT AGT TCA TCA GGC ACA TTT CCT TGT	288
Ile Asp Tyr Asn Ile Pro Cys Val Ser Ser Ser Gly Thr Phe Pro Cys	
85 90 95	
CCT CAA GAA GAT TCC TAT GGA AAC TGG GGA TGC AAA GGA ATG GGT GCT	336
Pro Gln Glu Asp Ser Tyr Gly Asn Trp Gly Cys Lys Gly Met Gly Ala	
100 105 110	
TGT TCT AAT AGT CAA GGA ATT GCA TAC TGG AGT ACT GAT TTA TTT GGT	384
Cys Ser Asn Ser Gln Gly Ile Ala Tyr Trp Ser Thr Asp Leu Phe Gly	
115 120 125	

TTC TAT ACT ACC CCA ACA AAC GTA ACC CTA GAA ATG ACA GGT TAT TTT	432
Phe Tyr Thr Thr Pro Thr Asn Val Thr Leu Glu Met Thr Gly Tyr Phe	
130 135 140	
TTA CCA CCA CAG ACG GGT TCT TAC ACA TTC AAG TTT GCT ACA GTT GAC	480
Leu Pro Pro Gln Thr Gly Ser Tyr Thr Phe Lys Phe Ala Thr Val Asp	
145 150 155 160	
GAC TCT GCA ATT CTA TCA GTA GGT GGT GCA ACC GCG TTC AAC TGT TGT	528
Asp Ser Ala Ile Leu Ser Val Gly Gly Ala Thr Ala Phe Asn Cys Cys	
165 170 175	
GCT CAA CAG CAA CCG CCG ATC ACA TCA ACG AAC TTT ACC ATT GAC GGT	576
Ala Gln Gln Gln Pro Pro Ile Thr Ser Thr Asn Phe Thr Ile Asp Gly	
180 185 190	
ATC AAG CCA TGG GGT GGA AGT TTG CCA CCT AAT ATC GAA GGA ACC GTC	624
Ile Lys Pro Trp Gly Gly Ser Leu Pro Pro Asn Ile Glu Gly Thr Val	
195 200 205	
TAT ATG TAC GCT GGC TAC TAT TAT CCA ATG AAG GTT GTT TAC TCG AAC	672
Tyr Met Tyr Ala Gly Tyr Tyr Tyr Pro Met Lys Val Val Tyr Ser Asn	
210 215 220	
GCT GTT TCT TGG GGT ACA CTT CCA ATT AGT GTG ACA CTT CCA GAT GGT	720
Ala Val Ser Trp Gly Thr Leu Pro Ile Ser Val Thr Leu Pro Asp Gly	
225 230 235 240	
ACC ACT GTA AGT GAT GAC TTC GAA GGG TAC GTC TAT TCC TTT GAC GAT	768
Thr Thr Val Ser Asp Asp Phe Glu Gly Tyr Val Tyr Ser Phe Asp Asp	
245 250 255	
GAC CTA AGT CAA TCT AAC TGT ACT GTC CCT GAC CCT TCA AAT TAT GCT	816
Asp Leu Ser Gln Ser Asn Cys Thr Val Pro Asp Pro Ser Asn Tyr Ala	
260 265 270	
GTC AGT ACC ACT ACA ACT ACA ACG GAA CCA TGG ACC GGT ACT TTC ACT	864
Val Ser Thr Thr Thr Thr Thr Thr Glu Pro Trp Thr Gly Thr Phe Thr	
275 280 285	
TCT ACA TCT ACT GAA ATG ACC ACC GTC ACC GGT ACC AAC GGC GTT CCA	912
Ser Thr Ser Thr Glu Met Thr Thr Val Thr Gly Thr Asn Gly Val Pro	
290 295 300	

TAT CCT AGC AAT GGA ACT TCT GTG ATT TCT TCC TCA GTA ATT TCT TCC	1488
Tyr Pro Ser Asn Gly Thr Ser Val Ile Ser Ser Ser Val Ile Ser Ser	
485 490 495	
TCA GTC ACT TCT TCT CTA TTC ACT TCT TCT CCA GTC ATT TCT TCC TCA	1536
Ser Val Thr Ser Ser Leu Phe Thr Ser Ser Pro Val Ile Ser Ser Ser	
500 505 510	
GTC ATT TCT TCT TCT ACA ACA ACC TCC ACT TCT ATA TTT TCT GAA TCA	1584
Val Ile Ser Ser Ser Thr Thr Thr Ser Thr Ser Ile Phe Ser Glu Ser	
515 520 525	
TCT AAA TCA TCC GTC ATT CCA ACC AGT AGT TCC ACC TCT GGT TCT TCT	1632
Ser Lys Ser Ser Val Ile Pro Thr Ser Ser Ser Thr Ser Gly Ser Ser	
530 535 540	
GAG AGC GAA ACG AGT TCA GCT GGT TCT GTC TCT TCT TCC TCT TTT ATC	1680
Glu Ser Glu Thr Ser Ser Ala Gly Ser Val Ser Ser Ser Ser Phe Ile	
545 550 555 560	
TCT TCT GAA TCA TCA AAA TCT CCT ACA TAT TCT TCT TCA TCA TTA CCA	1728
Ser Ser Glu Ser Ser Lys Ser Pro Thr Tyr Ser Ser Ser Ser Leu Pro	
565 570 575	
CTT GTT ACC AGT GCG ACA ACA AGC CAG GAA ACT GCT TCT TCA TTA CCA	1776
Leu Val Thr Ser Ala Thr Thr Ser Gln Glu Thr Ala Ser Ser Leu Pro	
580 585 590	
CCT GCT ACC ACT ACA AAA ACG AGC GAA CAA ACC ACT TTG GTT ACC GTG	1824
Pro Ala Thr Thr Thr Lys Thr Ser Glu Gln Thr Thr Leu Val Thr Val	
595 600 605	
ACA TCC TGC GAG TCT CAT GTG TGC ACT GAA TCC ATC TCC CCT GCG ATT	1872
Thr Ser Cys Glu Ser His Val Cys Thr Glu Ser Ile Ser Pro Ala Ile	
610 615 620	
GTT TCC ACA GCT ACT GTT ACT GTT AGC GGC GTC ACA ACA GAG TAT ACC	1920
Val Ser Thr Ala Thr Val Thr Val Ser Gly Val Thr Thr Glu Tyr Thr	
625 630 635 640	
ACA TGG TGC CCT ATT TCT ACT ACA GAG ACA ACA AAG CAA ACC AAA GGG	1968
Thr Trp Cys Pro Ile Ser Thr Thr Glu Thr Thr Lys Gln Thr Lys Gly	
645 650 655	

ACA ACA GAG CAA ACC ACA GAA ACA ACA AAA CAA ACC ACG GTA GTT ACA	2016
Thr Thr Glu Gln Thr Thr Glu Thr Thr Lys Gln Thr Thr Val Val Thr	
660 665 670	
ATT TCT TCT TGT GAA TCT GAC GTA TGC TCT AAG ACT GCT TCT CCA GCC	2064
Ile Ser Ser Cys Glu Ser Asp Val Cys Ser Lys Thr Ala Ser Pro Ala	
675 680 685	
ATT GTA TCT ACA AGC ACT GCT ACT ATT AAC GGC GTT ACT ACA GAA TAC	2112
Ile Val Ser Thr Ser Thr Ala Thr Ile Asn Gly Val Thr Thr Glu Tyr	
690 695 700	
ACA ACA TGG TGT CCT ATT TCC ACC ACA GAA TCG AGG CAA CAA ACA ACG	2160
Thr Thr Trp Cys Pro Ile Ser Thr Thr Glu Ser Arg Gln Gln Thr Thr	
705 710 715 720	
CTA GTT ACT GTT ACT TCC TGC GAA TCT GGT GTG TGT TCC GAA ACT GCT	2208
Leu Val Thr Val Thr Ser Cys Glu Ser Gly Val Cys Ser Glu Thr Ala	
725 730 735	
TCA CCT GCC ATT GTT TCG ACG GCC ACG GCT ACT GTG AAT GAT GTT GTT	2256
Ser Pro Ala Ile Val Ser Thr Ala Thr Ala Thr Val Asn Asp Val Val	
740 745 750	
ACG GTC TAT CCT ACA TGG AGG CCA CAG ACT GCG AAT GAA GAG TCT GTC	2304
Thr Val Tyr Pro Thr Trp Arg Pro Gln Thr Ala Asn Glu Glu Ser Val	
755 760 765	
AGC TCT AAA ATG AAC AGT GCT ACC GGT GAG ACA ACA ACC AAT ACT TTA	2352
Ser Ser Lys Met Asn Ser Ala Thr Gly Glu Thr Thr Thr Asn Thr Leu	
770 775 780	
GCT GCT GAA ACG ACT ACC AAT ACT GTA GCT GCT GAG ACG ATT ACC AAT	2400
Ala Ala Glu Thr Thr Thr Asn Thr Val Ala Ala Glu Thr Ile Thr Asn	
785 790 795 800	
ACT GGA GCT GCT GAG ACG AAA ACA GTA GTC ACC TCT TCG CTT TCA AGA	2448
Thr Gly Ala Ala Glu Thr Lys Thr Val Val Thr Ser Ser Leu Ser Arg	
805 810 815	
TCT AAT CAC GCT GAA ACA CAG ACG GCT TCC GCG ACC GAT GTG ATT GGT	2496
Ser Asn His Ala Glu Thr Gln Thr Ala Ser Ala Thr Asp Val Ile Gly	
820 825 830	

64

CAC AGC AGT AGT GTT GTT TCT GTA TCC GAA ACT GGC AAC ACC AAG AGT	2544
His Ser Ser Ser Val Val Ser Val Ser Glu Thr Gly Asn Thr Lys Ser	
835 840 845	
CTA ACA AGT TCC GGG TTG AGT ACT ATG TCG CAA CAG CCT CGT AGC ACA	2592
Leu Thr Ser Ser Gly Leu Ser Thr Met Ser Gln Gln Pro Arg Ser Thr	
850 855 860	
CCA GCA AGC AGC ATG GTA GGA TAT AGT ACA GCT TCT TTA GAA ATT TCA	2640
Pro Ala Ser Ser Met Val Gly Tyr Ser Thr Ala Ser Leu Glu Ile Ser	
865 870 875 880	
ACG TAT GCT GGC AGT GCA ACA GCT TAC TGG CCG GTA GTG GTT TAA	2686
Thr Tyr Ala Gly Ser Ala Thr Ala Tyr Trp Pro Val Val Val	
885 890 895	

(2) INFORMATION FOR SEQ ID NO: 22:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 894 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 22:

Met Thr Met Pro His Arg Tyr Met Phe Leu Ala Val Phe Thr Leu Leu	
1 5 10 15	
Ala Leu Thr Ser Val Ala Ser Gly Ala Thr Glu Ala Cys Leu Pro Ala	
20 25 30	
Gly Gln Arg Lys Ser Gly Met Asn Ile Asn Phe Tyr Gln Tyr Ser Leu	
35 40 45	
Lys Asp Ser Ser Thr Tyr Ser Asn Ala Ala Tyr Met Ala Tyr Gly Tyr	
50 55 60	
Ala Ser Lys Thr Lys Leu Gly Ser Val Gly Gly Gln Thr Asp Ile Ser	
65 70 75 80	
Ile Asp Tyr Asn Ile Pro Cys Val Ser Ser Ser Gly Thr Phe Pro Cys	
85 90 95	

65

Pro Gln Glu Asp Ser Tyr Gly Asn Trp Gly Cys Lys Gly Met Gly Ala
 100 105 110

Cys Ser Asn Ser Gln Gly Ile Ala Tyr Trp Ser Thr Asp Leu Phe Gly
 115 120 125

Phe Tyr Thr Thr Pro Thr Asn Val Thr Leu Glu Met Thr Gly Tyr Phe
 130 135 140

Leu Pro Pro Gln Thr Gly Ser Tyr Thr Phe Lys Phe Ala Thr Val Asp
 145 150 155 160

Asp Ser Ala Ile Leu Ser Val Gly Gly Ala Thr Ala Phe Asn Cys Cys
 165 170 175

Ala Gln Gln Gln Pro Pro Ile Thr Ser Thr Asn Phe Thr Ile Asp Gly
 180 185 190

Ile Lys Pro Trp Gly Gly Ser Leu Pro Pro Asn Ile Glu Gly Thr Val
 195 200 205

Tyr Met Tyr Ala Gly Tyr Tyr Tyr Pro Met Lys Val Val Tyr Ser Asn
 210 215 220

Ala Val Ser Trp Gly Thr Leu Pro Ile Ser Val Thr Leu Pro Asp Gly
 225 230 235 240

Thr Thr Val Ser Asp Asp Phe Glu Gly Tyr Val Tyr Ser Phe Asp Asp
 245 250 255

Asp Leu Ser Gln Ser Asn Cys Thr Val Pro Asp Pro Ser Asn Tyr Ala
 260 265 270

Val Ser Thr Thr Thr Thr Thr Thr Glu Pro Trp Thr Gly Thr Phe Thr
 275 280 285

Ser Thr Ser Thr Glu Met Thr Thr Val Thr Gly Thr Asn Gly Val Pro
 290 295 300

Thr Asp Glu Thr Val Ile Val Ile Arg Thr Pro Thr Ser Glu Gly Leu
 305 310 315 320

Ile Ser Thr Thr Thr Glu Pro Trp Thr Gly Thr Phe Thr Ser Thr Ser
 325 330 335

Thr Glu Val Thr Thr Ile Thr Gly Thr Asn Gly Gln Pro Thr Asp Glu
 340 345 350

Thr Val Ile Val Ile Arg Thr Pro Thr Ser Glu Gly Leu Ile Ser Thr
 355 360 365

Thr Thr Glu Pro Trp Thr Gly Thr Phe Thr Ser Thr Ser Thr Glu Met
 370 375 380

Thr Thr Val Thr Gly Thr Asn Gly Gln Pro Thr Asp Glu Thr Val Ile
 385 390 395 400

Val Ile Arg Thr Pro Thr Ser Glu Gly Leu Val Thr Thr Thr Thr Glu
 405 410 415

Pro Trp Thr Gly Thr Phe Thr Ser Thr Ser Thr Glu Met Ser Thr Val
 420 425 430

Thr Gly Thr Asn Gly Leu Pro Thr Asp Glu Thr Val Ile Val Val Lys
 435 440 445

Thr Pro Thr Thr Ala Ile Ser Ser Ser Leu Ser Ser Ser Ser Ser Gly
 450 455 460

Gln Ile Thr Ser Ser Ile Thr Ser Ser Arg Pro Ile Ile Thr Pro Phe
 465 470 475 480

Tyr Pro Ser Asn Gly Thr Ser Val Ile Ser Ser Ser Val Ile Ser Ser
 485 490 495

Ser Val Thr Ser Ser Leu Phe Thr Ser Ser Pro Val Ile Ser Ser Ser
 500 505 510

Val Ile Ser Ser Ser Thr Thr Thr Ser Thr Ser Ile Phe Ser Glu Ser
 515 520 525

Ser Lys Ser Ser Val Ile Pro Thr Ser Ser Ser Thr Ser Gly Ser Ser
 530 535 540

Glu Ser Glu Thr Ser Ser Ala Gly Ser Val Ser Ser Ser Ser Phe Ile
 545 550 555 560

Ser Ser Glu Ser Ser Lys Ser Pro Thr Tyr Ser Ser Ser Ser Leu Pro
 565 570 575

Leu Val Thr Ser Ala Thr Thr Ser Gln Glu Thr Ala Ser Ser Leu Pro
 580 585 590

Pro Ala Thr Thr Thr Lys Thr Ser Glu Gln Thr Thr Leu Val Thr Val
 595 600 605

Thr Ser Cys Glu Ser His Val Cys Thr Glu Ser Ile Ser Pro Ala Ile
 610 615 620

Val Ser Thr Ala Thr Val Thr Val Ser Gly Val Thr Thr Glu Tyr Thr
 625 630 635 640

Thr Trp Cys Pro Ile Ser Thr Thr Glu Thr Thr Lys Gln Thr Lys Gly
 645 650 655

Thr Thr Glu Gln Thr Thr Glu Thr Thr Lys Gln Thr Thr Val Val Thr
 660 665 670

Ile Ser Ser Cys Glu Ser Asp Val Cys Ser Lys Thr Ala Ser Pro Ala
 675 680 685

Ile Val Ser Thr Ser Thr Ala Thr Ile Asn Gly Val Thr Thr Glu Tyr
 690 695 700

Thr Thr Trp Cys Pro Ile Ser Thr Thr Glu Ser Arg Gln Gln Thr Thr
 705 710 715 720

Leu Val Thr Val Thr Ser Cys Glu Ser Gly Val Cys Ser Glu Thr Ala
 725 730 735

Ser Pro Ala Ile Val Ser Thr Ala Thr Ala Thr Val Asn Asp Val Val
 740 745 750

Thr Val Tyr Pro Thr Trp Arg Pro Gln Thr Ala Asn Glu Glu Ser Val
 755 760 765

Ser Ser Lys Met Asn Ser Ala Thr Gly Glu Thr Thr Thr Asn Thr Leu
 770 775 780

Ala Ala Glu Thr Thr Thr Asn Thr Val Ala Ala Glu Thr Ile Thr Asn
 785 790 795 800

Thr Gly Ala Ala Glu Thr Lys Thr Val Val Thr Ser Ser Leu Ser Arg
 805 810 815

68

Ser Asn His Ala Glu Thr Gln Thr Ala Ser Ala Thr Asp Val Ile Gly
 820 825 830

His Ser Ser Ser Val Val Ser Val Ser Glu Thr Gly Asn Thr Lys Ser
 835 840 845

Leu Thr Ser Ser Gly Leu Ser Thr Met Ser Gln Gln Pro Arg Ser Thr
 850 855 860

Pro Ala Ser Ser Met Val Gly Tyr Ser Thr Ala Ser Leu Glu Ile Ser
 865 870 875 880

Thr Tyr Ala Gly Ser Ala Thr Ala Tyr Trp Pro Val Val Val
 885 890

(2) INFORMATION FOR SEQ ID NO: 23:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 30 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(vii) IMMEDIATE SOURCE:

- (B) CLONE: primer pcrflol

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 23:

GAATTCGCTA GCAATTATGC TGTCAGTACC

30

(2) INFORMATION FOR SEQ ID NO: 24:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 24 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(vii) IMMEDIATE SOURCE:

(B) CLONE: Part non-coding sequence FLO1

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 24:

AGTGGTACTG ACAGCATAAT TTGA

24

(2) INFORMATION FOR SEQ ID NO: 25:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 24 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(vii) IMMEDIATE SOURCE:

(B) CLONE: Part coding sequence FLO1

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 25:

AATAAAATTC GCGTTCTTTT TACG

24

(2) INFORMATION FOR SEQ ID NO: 26:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 31 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(vii) IMMEDIATE SOURCE:

(B) CLONE: primer pcrflo2

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 26:

GAGCTCAAGC TTCGTAAAAA GAACGCGAAT T

31

CLAIMS

1. A method for immobilizing an enzyme, comprising the use of recombinant DNA techniques for producing an enzyme or a functional part thereof linked to the cell wall of a host cell, preferably a microbial cell, and whereby the enzyme or functional fragment thereof is localized at the exterior of the cell wall.
2. The method of claim 1, wherein the enzyme or the functional part thereof is immobilized by linking to the C-terminal part of a protein that ensures anchoring in the cell wall.
3. A recombinant polynucleotide comprising a structural gene encoding a protein providing catalytic activity and at least a part of a gene encoding a protein capable of anchoring in a eukaryotic or prokaryotic cell wall, said part encoding at least the C-terminal part of said anchoring protein.
4. The polynucleotide of claim 3, further comprising a sequence encoding a signal peptide ensuring secretion of the expression product of the polynucleotide.
5. The polynucleotide of claim 4, wherein the signal peptide is derived from a protein selected from the group consisting of glycosyl-phosphatidyl-inositol (GPI) anchoring protein, α -factor, α -agglutinin, invertase or inulinase, α -amylase of *Bacillus*, and proteinases of lactic acid bacteria.
6. The polynucleotide of any of claims 3-5, wherein the protein capable of anchoring in the cell wall is selected from the group consisting of α -agglutinin, AGA1, FLO1, Major Cell Wall Protein of lower eukaryotes, and proteinases of lactic acid bacteria.
7. The polynucleotide of any of claims 3-6, operably linked to a promoter, preferably an inducible promoter.

8. The polynucleotide of any of claims 3-7, wherein the protein providing catalytic activity is a hydrolytic enzyme, e.g. a lipase.
9. The polynucleotide of any of claims 3-7, wherein the protein providing catalytic activity is an oxidoreductase, e.g. an oxidase.
10. A recombinant vector comprising a polynucleotide as claimed in any of claims 3-9.
11. The recombinant vector of claim 10, wherein the protein providing catalytic activity exhibits said catalytic activity when present in a multimeric form, said vector further comprising a second polynucleotide comprising a structural gene encoding the same protein providing catalytic activity combined with a sequence encoding a signal peptide ensuring secretion of the expression product of said second polynucleotide, said second polynucleotide being operably linked to a regulatable promoter, preferably an inducible or repressible promoter.
12. A chimeric protein encoded by a polynucleotide as claimed in any of claims 3-9.
13. A host cell, preferably a microorganism, containing a polynucleotide as claimed in any of claims 3-9 or a vector as claimed in claim 10 or 11.
14. A host cell, preferably a microorganism, containing a polynucleotide as claimed in any of claims 3-9 or a vector as claimed in claim 10, wherein the protein providing catalytic activity exhibits said catalytic activity when present in a multimeric form, said microorganism further comprising a second polynucleotide comprising a structural gene encoding the same protein providing catalytic activity combined with a sequence encoding a signal peptide ensuring secretion of the expression product of said second polynucleotide, said second polynucleotide being operably linked to a regulatable promoter, preferably an inducible or repressible promoter and said second polynucleotide being present either in another vector or in the chromosome of said microorganism.

15. The host cell or microorganism of claim 13 or 14, having at least one of said polynucleotides integrated in its chromosome.
16. A host cell, preferably a microorganism, having a protein as claimed in claim 12 immobilized on its cell wall.
17. The host cell or microorganism of any of claims 13-16, which is a lower eukaryote, in particular a yeast.
18. A process for carrying out an enzymatic process by using an immobilized catalytically active protein, wherein a substrate for said catalytically active protein is contacted with a host cell or microorganism as claimed in any of claims 13-17.

* * * * *

FIGURE 1, 1/4

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DNA SEQUENCE OF ALPHA-AGGLUTENIN:

1	AAGCTTTAGG	TAAGGGAGGC	AGGGGGAAAA	GATACTGAAA
41	TGACGGAAAA	CGAGAATATG	GAGCAGGGAG	CAACTTTTAG
81	AGCTTTACCC	GTTAAAAGGT	CAAATCGAGG	CTTCCTGCCT
121	TTGTCTGATT	TTAGTAGTAC	CGGAAGGTTT	ATTACGCCCA
161	AGAACAGTGC	TTGAATTGAG	TTCTCGGGAC	ACGGGAAAGA
201	CAATGGAAGA	AAAATTTACA	TCAGTAGCC	TTATATATGA
241	AATGCTGCCA	AGCCACGTCT	TTATAAGTAG	ATAATGTCCC
281	ATGAGCTGAA	CTATGGGAAT	TTATGACGCA	GTTCAATTGTA
321	TATATATTAC	ATTAACCTCT	TAGTTTAACA	TCTGAATTGT
361	TTTATAAAAT	AACTTTTTTGA	ATTTTTTTTAT	GATCGCTTAG
401	TTAAGTCTAT	TATATCAGGT	TTTTTTCATTC	ATCATAATTG
441	TTCGTTAAAT	ATGAGTATAT	TTAAATACAG	GAATTAGTAT
481	CATTTGCAGT	CACGAAAAGG	GCCGTTTCAT	AGAGAGTTTT
521	CTTAATAAAG	TTGAGGGTTT	CCGTGATAGT	TTTGAGGGGT
561	TGTTTGAAC	AGATTTACGC	TTACCTTTCA	ACTGATTAAT
601	TTTTTCAGCG	GGCTTATCAT	AATCATCCAT	CATAGCAGTC
641	TTTCTGGACT	TCGTCGAGGA	CTGGCTTTCT	GAATTTTGAC
681	GGTCCCTATT	AGCTCCAGTT	GGAGGAATTG	AGTTACCTAC
721	AACTGGCAAG	AGGTCTTTGT	TTGGATTCAA	AATAGGACTT
761	TGTGGTAGCA	GTTTGGTTTT	ATTCAATCTA	AAGATATGAG
801	AAACAGGTTT	TAAGTAAATC	GATACTATTG	TACCAATGTT
841	TAGCTCCAAT	TCCTCCAAAA	CGGTGGGATC	TAATTTTGTG
881	TTCATTTCTA	TTAGTGGCAA	CTCTCCGTCC	AGTACTGATT
921	TTAAAGATT	AAAAGTTATC	GCGTTTGATA	TACGAGACGT
961	TTTCGTTAAT	GACAGCAATC	TCCAATACAT	CAGTGTTTTA
1001	TCTCTTAAGT	CAGGATTATT	TTCGTGATCG	GTGCATCCTT
1041	TTAATAAATC	CATACAAAGT	TCTTCAGTTT	CCTTTGTAGG
1081	ATTTCTGATG	AAGAATTTTA	TTGCTGAGTT	CAGAATGGAA
1121	AATTGCACTT	CTAGCGTCTC	ATTAAACATG	TTTGAGGAAA
1161	AAACTCTAAA	TAACCTCAGG	TAGTTTGGAA	TTACATCCGA
1201	ATATTGCGTT	ATTATCCAGA	TCATAGCGTT	TTTTGATTCA
1241	GGTTCCTGTA	CAACTTCAGT	GTGTTTGACT	AGTTCTGTTA
1281	CGTTTGCTTT	AAAATTATTG	GGATATTTCC	TCAAAATATT
1321	TCTGAAAACC	GAAATAATCT	CCTGGACGAC	ATAATCAACA
1361	CCGAATTCTA	ACAAATCTAG	TAGCACAGCG	ACACAATCGT
1401	GTACAGAGTC	TTCATCTAGC	TTAACAGCGA	GATTACCAAT
1441	GGCTCTGACT	GATTTCCCTG	ACATTTGAAT	ATCAATATCT
1481	GTAGCATATT	GTTCCAATC	TTCTAGAATT	CTTGGTAATG
1521	TTTCCTTGTT	AGCTAAAAGA	TATAAACACT	CTAATTTCTG
1561	GTCTTTGATG	TATATGGGGT	CATTGTACTC	GATGAAAAAA

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FIGURE 1, 2/4

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1601	TACGAAATGT	CTAGCCTGAG	TAGAGATGAC	TCCCTACTCA
1641	ATAAAAGAAG	AATAACGTTT	CTTAATACTA	AAAATTGTAA
1681	TTCAGGCGGC	TTATCTAACA	AAGCTATTAC	AGAGTTAGAT
1721	AGCTTTTTCGG	CTAGAGTTTC	TTTGATGACG	TCAACATAAT
1761	TCAACAAGTA	CATGATGAAT	TTTAAAGAGT	TCAACACTAC
1801	GTATGTGTTT	ACTTGTTGCA	GGTACGGTAA	AGCTAGTTTCG
1841	ATCATTTTCAT	GGGTATCCAA	ATAATGCTGC	GGCACAACCG
1881	AAGTCGTCAA	AACTTCCAAA	ACAGTAGCCT	TATTCCACTC
1921	ATTTAATTTCG	GGTAAAAGTT	CTAGCATGTC	AAAAGCGAGT
1961	TCCAAGGGAA	TCCTGAAGGT	TCCATGTTAG	CGTTTTTTTC
2001	GTGAATGGAA	TATAAAGTAT	GTAATGCAGC	TACAATGACT
2041	TCTGGAGAGC	TCGACTGTGC	CTTTACAATG	TCATGTAGAA
2081	TGCTTGATAA	CCCCAATACC	CTTTCATGAT	CAATTTTCATC
2121	TAAATCCAAC	AGTGCGTAAA	TTGCTGTCCT	CGTCACTTGT
2161	TCAGGTGGAG	ACTTGTGATT	TACCAATGAA	ATGATACAGT
2201	CGAAGGCCTG	ATCAGATAGC	TCTTTCACCG	GGACTAATAC
2241	CAGAGTTCTT	AGTGCCATTA	TTTGTAAGTT	TTCATCTCTG
2281	CTTTTGAAAT	CGTCCATTAT	AAATGGCAAA	GCCTCTCTGG
2321	CCTGCTGAGG	TTTTAATGCG	CCGATCACCC	TAATATACTC
2361	ATGGCAAATT	CTTTTCACTT	CTAGATCATC	TTCAATTTGC
2401	CAAAATTTCA	AGAGCTCAGA	AAACAGAAGG	GACATTTTCGC
2441	CATAGTTTCC	TAGAACCAAA	TTGGCGATAA	TTTTTCTCAG
2481	AGCATTTTTTC	CTTCTTGTTA	TATTCGATTT	AAACTTTTTTT
2521	ACTCCAAAAT	GTTGCAGATC	TGTGACGATT	TCATTTGCTT
2561	TATATCTGGC	AAAAACTTTT	TGATCGGACA	TAAGCGAAAT
2601	ACGTCCTATT	AATGAAGTGA	ATGTTCTTGC	TGTATTCCCT
2641	TCTTGTGCAG	TAGATTAATT	CTGTTTCCAG	GCTGCGATAC
2681	TTTGATACCC	AATACTAAAA	GTTGATGATT	TGAACGATCT
2721	CCTATTTCCCT	CGCACATTTT	TGGAGCGATA	CCCGGAAGAC
2761	AGAATCGCGA	TGTTAAGAAA	ATAGTTCTGA	TGGCACTAAA
2801	GAGATCATGA	TTAAGGAAAG	GTAAGTGATA	TGCATGAATG
2841	GGAATAGGCT	TTCGAACTTG	ACGATTTAGT	TCCTTATTTT
2881	TATCCATCTA	ATCCTCCAAC	TTCAATAGGC	CTTATCTAGC
2921	TCAGAGCAGT	ATTTAATTGA	GAATAGTAGC	TTAATTGAAA
2961	CCTTACTAAA	AAAGTGTATG	GTTACATAAG	ATAAGGCGTT
3001	AAGAAGAGTA	TACATATGCA	TTATTCATTA	CCAAGACCAC
3041	TATGAATAGT	AATACCATAT	TTAGCTTTTG	AAACTCATGT
3081	TTTCTATTGT	GTTGTTTCAA	ATTCCTCTGT	TAGGCTCAAT
3121	TTAGGTTAAT	TAAATTATAA	AAAAATATAA	AAAATAAAGA
3161	AAGTTTATCC	ATCGGCACCT	CAATTCAATG	GAGTAAACAG
3201	TTTCAACACT	GAGTGGTGAA	ACATTGAACA	ACTACATGCA
3241	GTTTCCCGCC	ACGAGGCAAG	TGTAGGTCCT	TTGTCCATTT

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FIGURE 1, 3/4

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3281	CGCTTTGTTT	TGCAGGTCAT	TGATGACCTA	ATTAGGAAGG
3321	TAGAAGCCGC	TCCAGCTCAA	TAAGGAAATG	CTAAGGGTAC
3361	TCGCCTTTGG	TGTTTTIACCA	TACAATGGCA	GCTTTATGTC
3401	ACTTCATTCT	TCAGTAACGG	CGCTTAAATA	TTCCCAAAAA
3441	CGTTACAATG	GAATTGTTTG	ATCATGTAAC	GAAATGCAAT
3481	CTTCTAAAAA	AAAAGCCATG	TGAATCAAAA	AAAGATTCTT
3521	TTTAGCATAC	TATAAATATG	CAAAATGCCC	TCTATTTATT
3561	CTAGTAATCG	TCCATTCTCA	TATCTTCCTT	ATATCAGTCG
3601	CCTCGCTTAA	TATAGTCAGC	ACAAAAGGAA	CAACAATTCTG
3641	CCAGTTTTCA	AAATGTTTCA	TTTTCTCAAA	ATTATTCTGT
3681	GGCTTTTTTC	CTTGGCATTG	GCCTCTGCTA	TAAATATCAA
3721	CGATATCACA	TTTTCCAATT	TAGAAATTAC	TCCACTGACT
3761	GCAAATAAAC	AACCTGATCA	AGGTTGGACT	GCCACTTTTG
3801	ATTTTAGTAT	TGCAGATGCG	TCTTCCATTA	GGGAGGGCGA
3841	TGAATTCACA	TTATCAATGC	CACATGTTTA	TAGGATTAAG
3881	CTATTAAACT	CATCGCAAAC	AGCTACTATT	TCCTTAGCGG
3921	ATGGTACTGA	GGCTTTCAAA	TGCTATGTTT	CGCAACAGGC
3961	TGCATACTTG	TATGAAAATA	CTACTTTCAC	ATGTACTGCT
4001	CAAAATGACC	TGTCCTCCTA	TAATACGATT	GATGGATCCA
4041	TAACATTTTC	GCTAAATTTT	AGTGATGGTG	GTTCCAGCTA
4081	TGAATATGAG	TTAGAAAACG	CTAAGTTTTT	CAAATCTGGG
4121	CCAATGCTTG	TTAAACTTGG	TAATCAAATG	TCAGATGTGG
4161	TGAATTTCTGA	TCCTGCTGCT	TTTACAGAGA	ATGTTTTTCA
4201	CTCTGGGCGT	TCAACTGGTT	ACGGTTCTTT	TGAAAGTTAT
4241	CATTTGGGTA	TGTATTGTCC	AAACGGATAT	TTCCTGGGTG
4281	GTA CTGAGAA	GATTGATTAC	GACAGTTCCA	ATAACAATGT
4321	CGATTTGGAT	TGTTCTTCAG	TTCAGGTTTA	TTCATCCAAT
4361	GATTTTAATG	ATTGGTGGTT	CCCGCAAAGT	TACAATGATA
4401	CCAATGCTGA	CGTCACTTGT	TTTGGTAGTA	ATCTGTGGAT
4441	TACACTTGAC	GAAAAACTAT	ATGATGGGGA	AATGTTATGG
4481	GTTAATGCAT	TACAATCTCT	ACCCGCTAAT	GTAAACACAA
4521	TAGATCATGC	GTTAGAATTT	CAATACACAT	GCCTTGATAC
4561	CATAGCAAAT	ACTACGTACG	CTACGCAATT	CTCGACTACT
4601	AGGGAATTTA	TTGTTTATCA	GGGTCGGAAC	CTCGGTACAG
4641	CTAGCGCCAA	AAGCTCTTTT	ATCTCAACCA	CTACTACTGA
4681	TTTAACAAGT	ATAAACACTA	GTGCGTATTC	CACTGGATCC
4721	ATTTCCACAG	TAGAAACAGG	CAATCGAACT	ACATCAGAAG
4761	TGATCAGTCA	TGTGGTGACT	ACCAGCACAA	AACTGTCTCC
4801	AACTGCTACT	ACCAGCCTGA	CAATTGCACA	AACCAGTATC
4841	TATTCTACTG	ACTCAAATAT	CACAGTAGGA	ACAGATATTC
4881	ACACCACATC	AGAAGTGATT	AGTGATGTGG	AAACCATTAG
4921	CAGAGAAACA	GCTTCGACCG	TTGTAGCCGC	TCCAACCTCA

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FIGURE 1, 4/4

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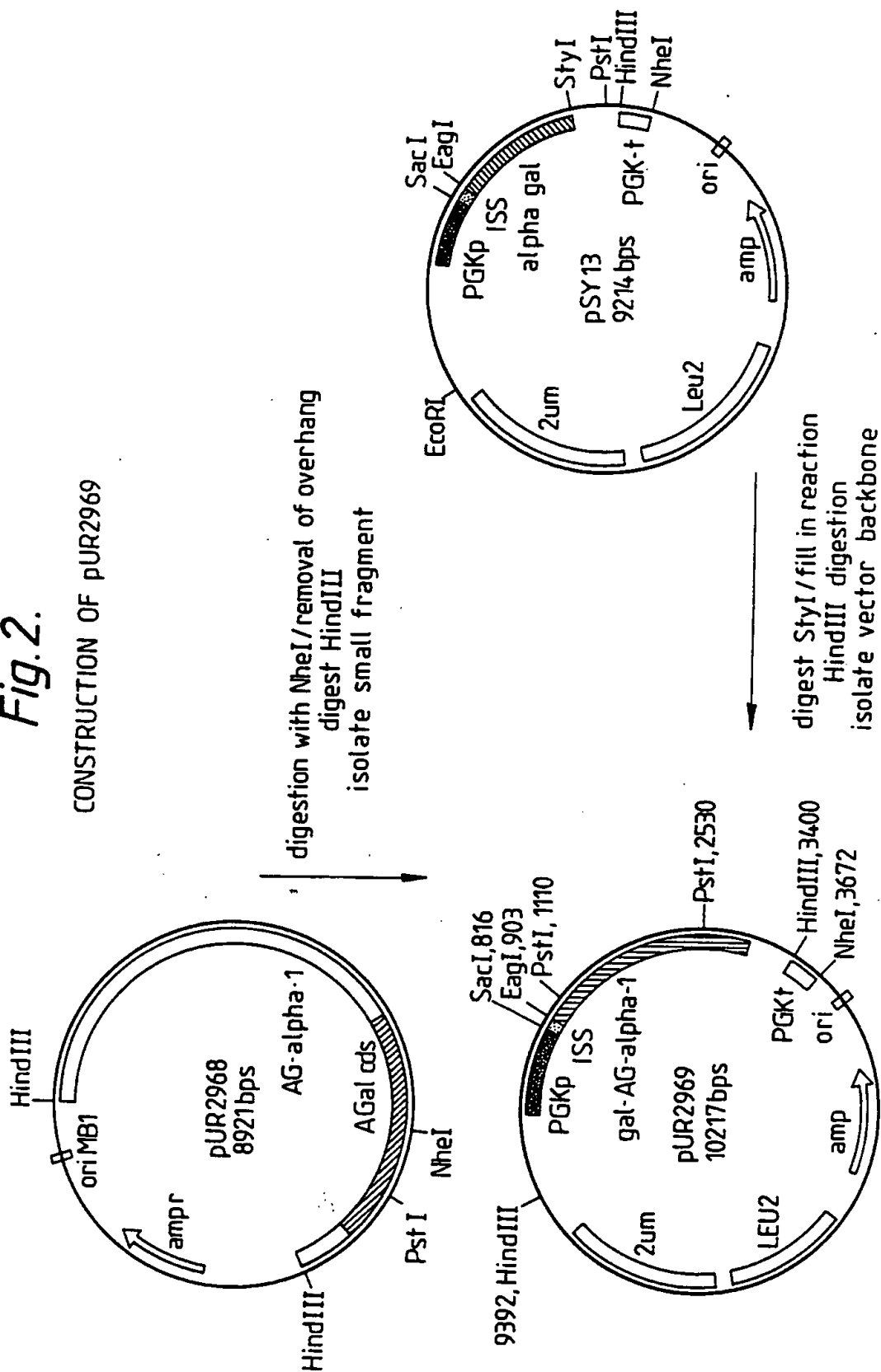
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5001	AATTT	TACATC	CTCTTCTTTC	GCAACAATCA	ACAGCACACC	
5041	AATAATCTCT	TCATCAGCAG	TATTTGAAAC	CTCAGATGCT		
5081	TCAATTGTCA	ATGTGCACAC	TGAAAATATC	ACGAATACTG		
5121	CTGCTGTTCC	ATCTGAAGAG	CCCACCTTTTG	TAAATGCCAC		
5161	GAGAAACTCC	TTAAATTTCCT	TCTGCAGCAG	CAAACAGCCA		
5201	TCCAGTCCCT	CATCTTATAC	GTCTTCCCCA	CTCGTATCGT		
5241	CCCTCTCCGT	AAGCAAAACA	TTACTAAGCA	CCAGTTTTAC		
5281	GCCTTCTGTG	CCAACATCTA	ATACATATAT	CAAAACGGAA		
5321	AATACGGGTT	ACTTTGAGCA	CACGGCTTTG	ACAACATCTT		
5361	CAGTTGGCCT	TAATTCTTTT	AGTGAAACAG	CACTCTCATC		
5401	TCAGGGAACG	AAAATTGACA	CCTTTTTTAGT	GTCATCCTTG		
5441	ATCGCATATC	CTTCTTCTGC	ATCAGGAAGC	CAATTGTCCG		
5481	GTATCCAACA	GAATTTCACA	TCAACTTCTC	TCATGATTTC		
5521	AACCTATGAA	GGTAAAGCGT	CTATATTTTT	CTCAGCTGAG		
5561	CTCGGTTCGA	TCATTTTTTCT	GCTTTTGTCG	TACCTGCTAT		
5601	TCTAAAACGG	GTACTGTACA	GTTAGTACAT	TGAGTCGAAA		
5641	TATACGAAAT	TATTGTTTCAT	AATTTTCATC	CTGGCTCTTT		
5681	TTTTCTTCAA	CCATAGTTAA	ATGGACAGTT	CATATCTTAA		
5721	ACTCTAATAA	TACTTTTCTA	GTTCTTATCC	TTTTCCGTCT		
5761	CACCGCAGAT	TTTATCATAG	TATTAAATTT	ATATTTTGTT		
5801	CGTAAAAAGA	AAAATTTGTG	AGCGTTACCG	CTCGTTTCAT		
5841	TACCCGAAGG	CTGTTTCAGT	AGACCACTGA	TTAAGTAAGT		
5881	AGATGAAAAA	ATTTCATCAC	CATGAAAGAG	TTCGATGAGA		
5921	GCTACTTTTT	CAAATGCTTA	ACAGCTAACC	GCCATTCAAT		
5961	AATGTTACGT	TCTCTTCATT	CTGCGGCTAC	GTTATCTAAC		
6001	AAGAGGTTTT	ACTCTCTCAT	ATCTCATTTCA	AATAGAAAGA		
6041	ACATAATCAA	AAAGCTT	6057			

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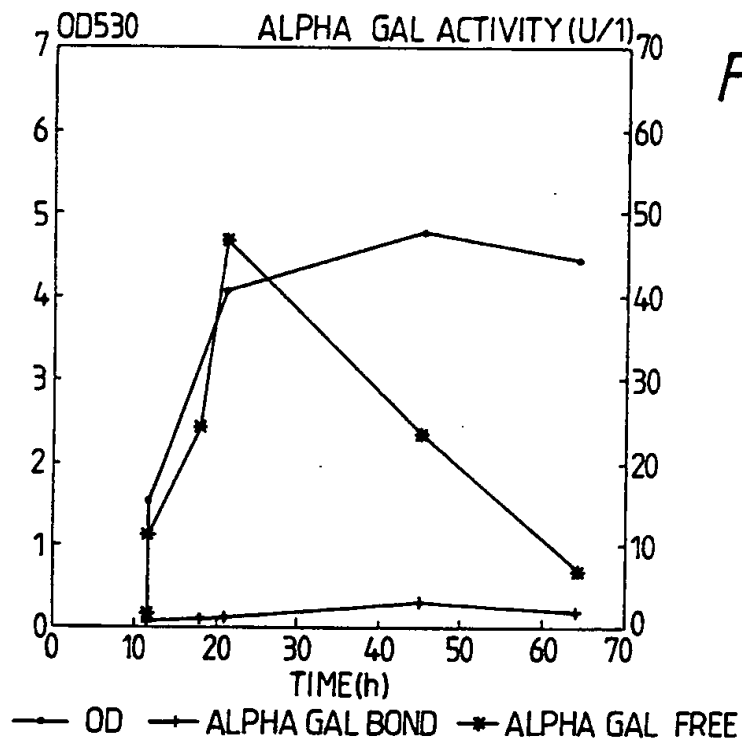
Fig. 2.

CONSTRUCTION OF pUR2969

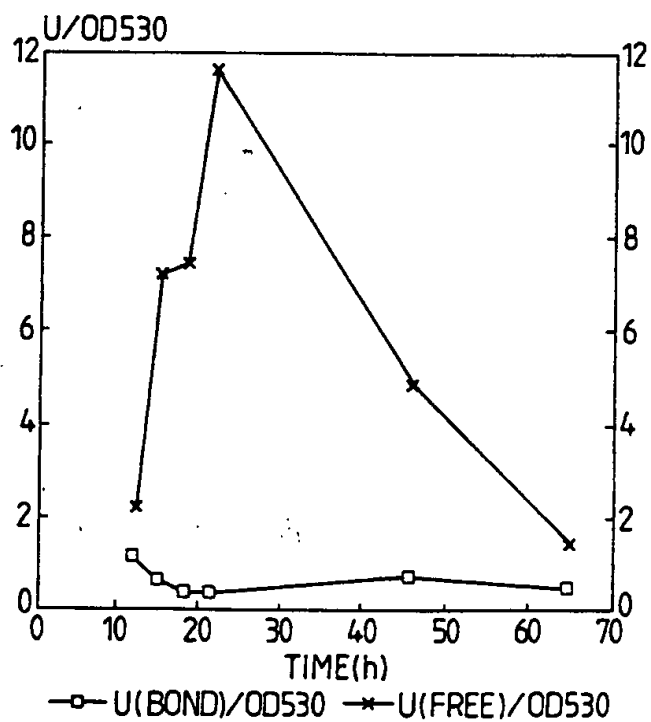


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ALPHA GALACTOSIDASE ACTIVITY MT302 /1c WITH pSY13

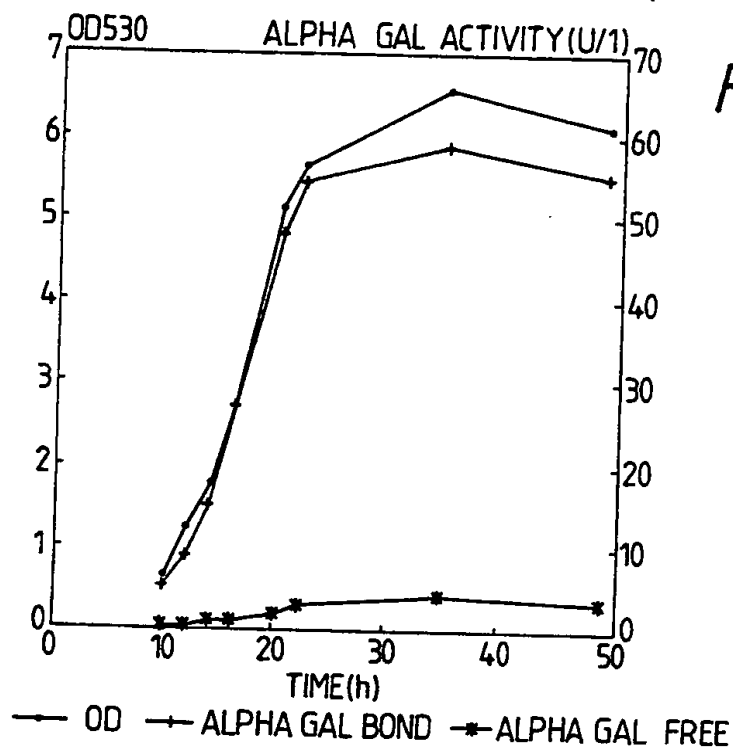


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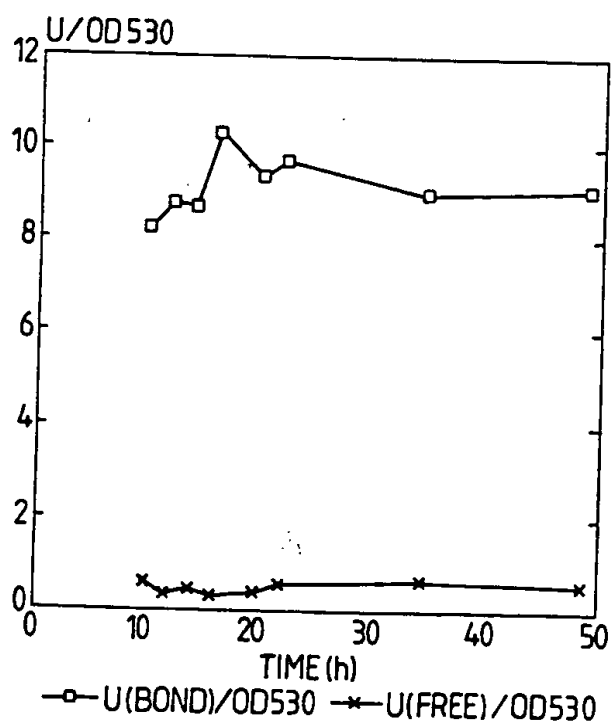


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ALPHA GALACTOSIDASE ACTIVITY MT302/1c WITH pUR2969

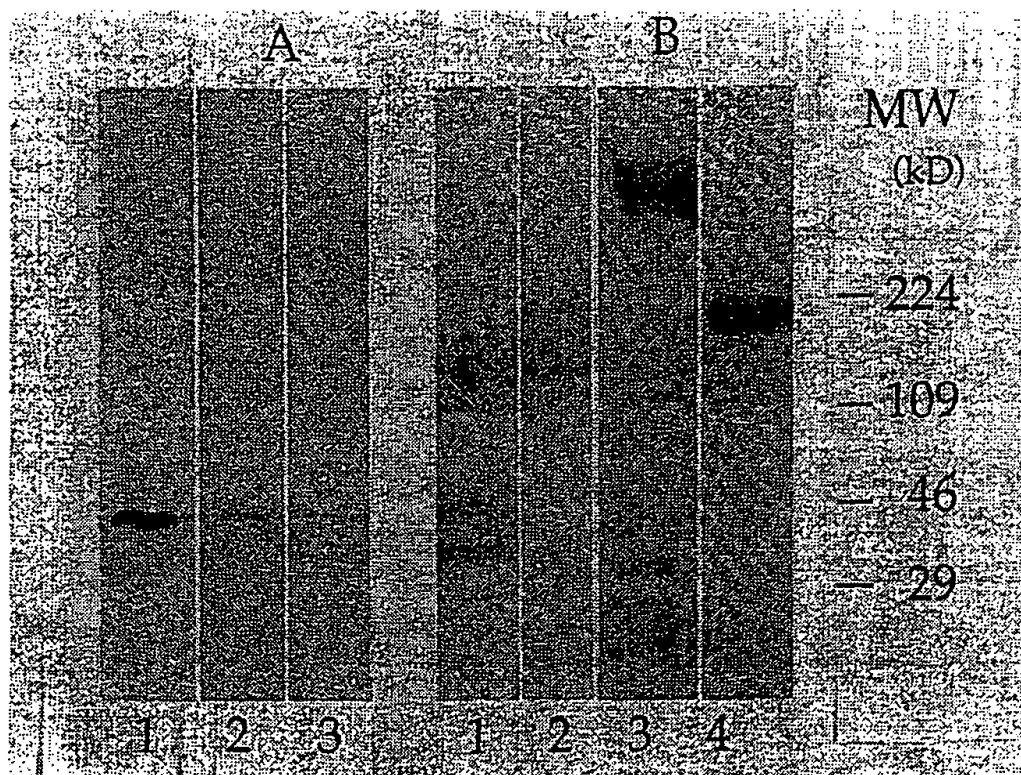


ALPHA GALACTOSIDASE ACTIVITY MT302/1c WITH pUR2969



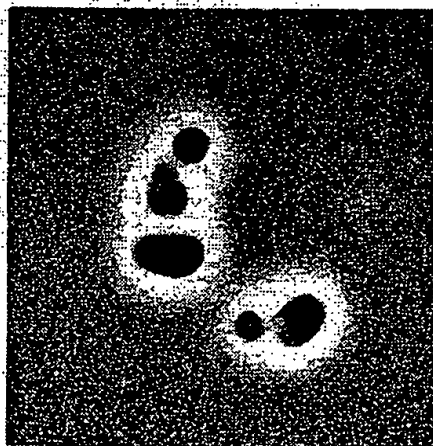
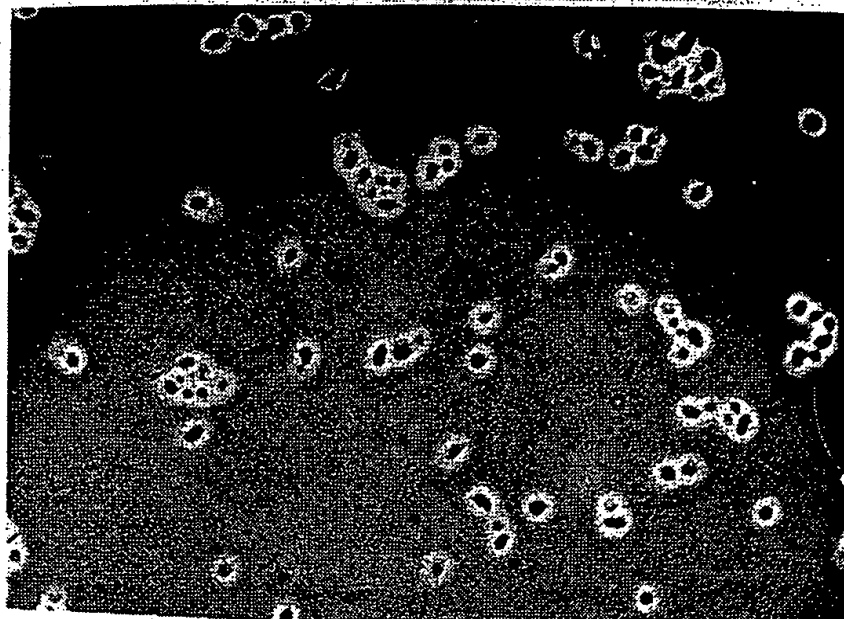
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Fig. 5.



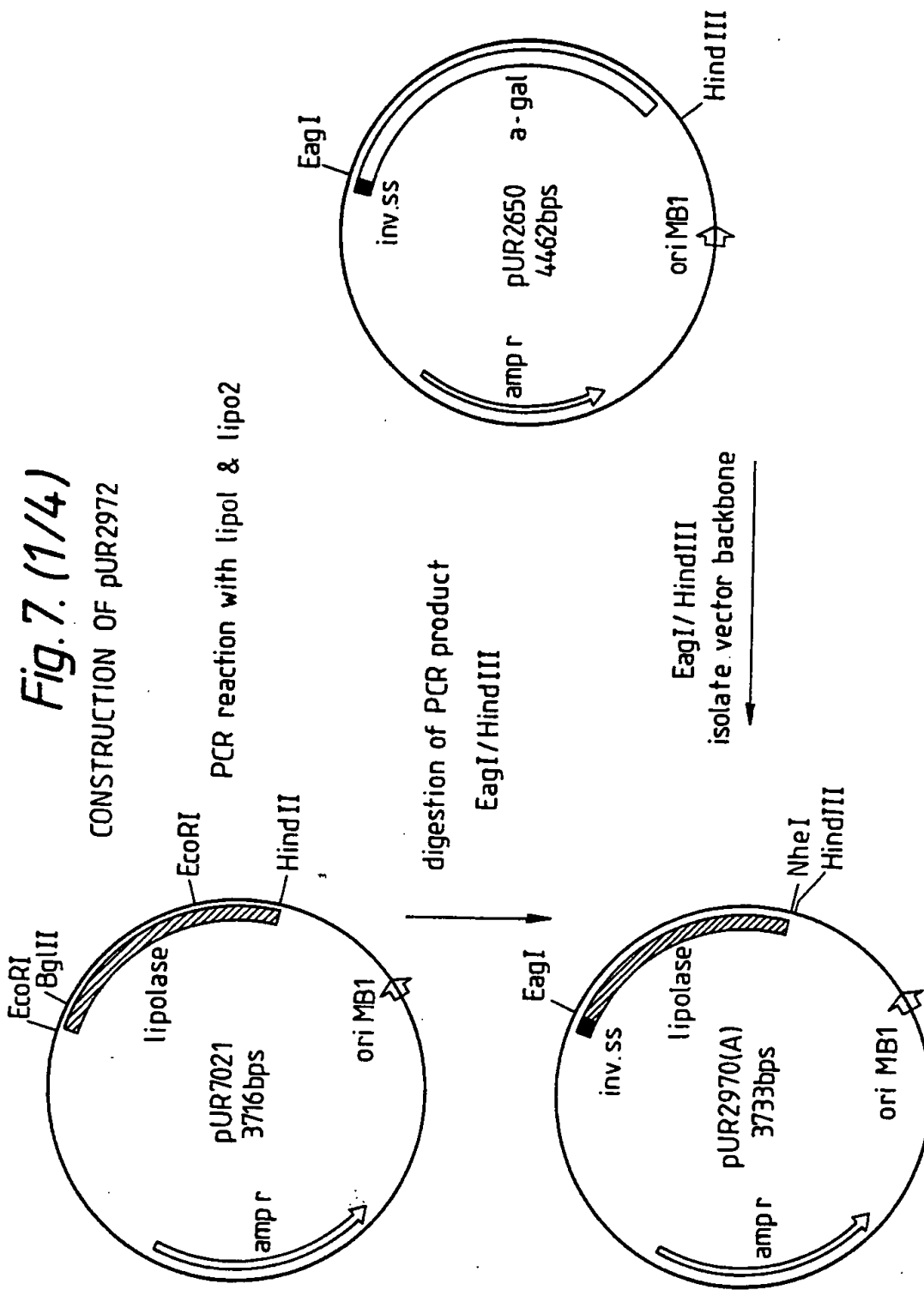
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Fig. 6. (1/2)



(2/2)

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Fig. 7 (2/4)

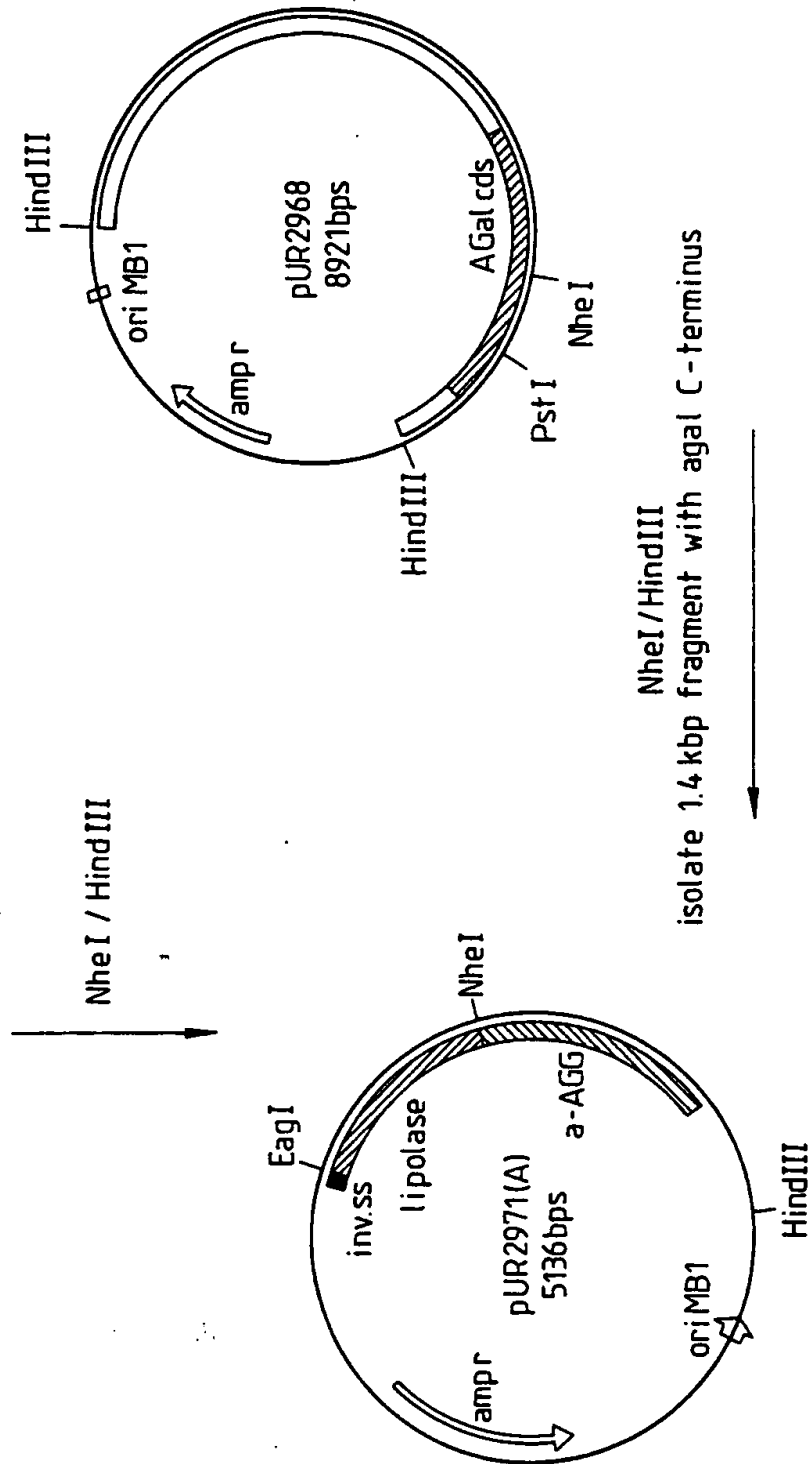
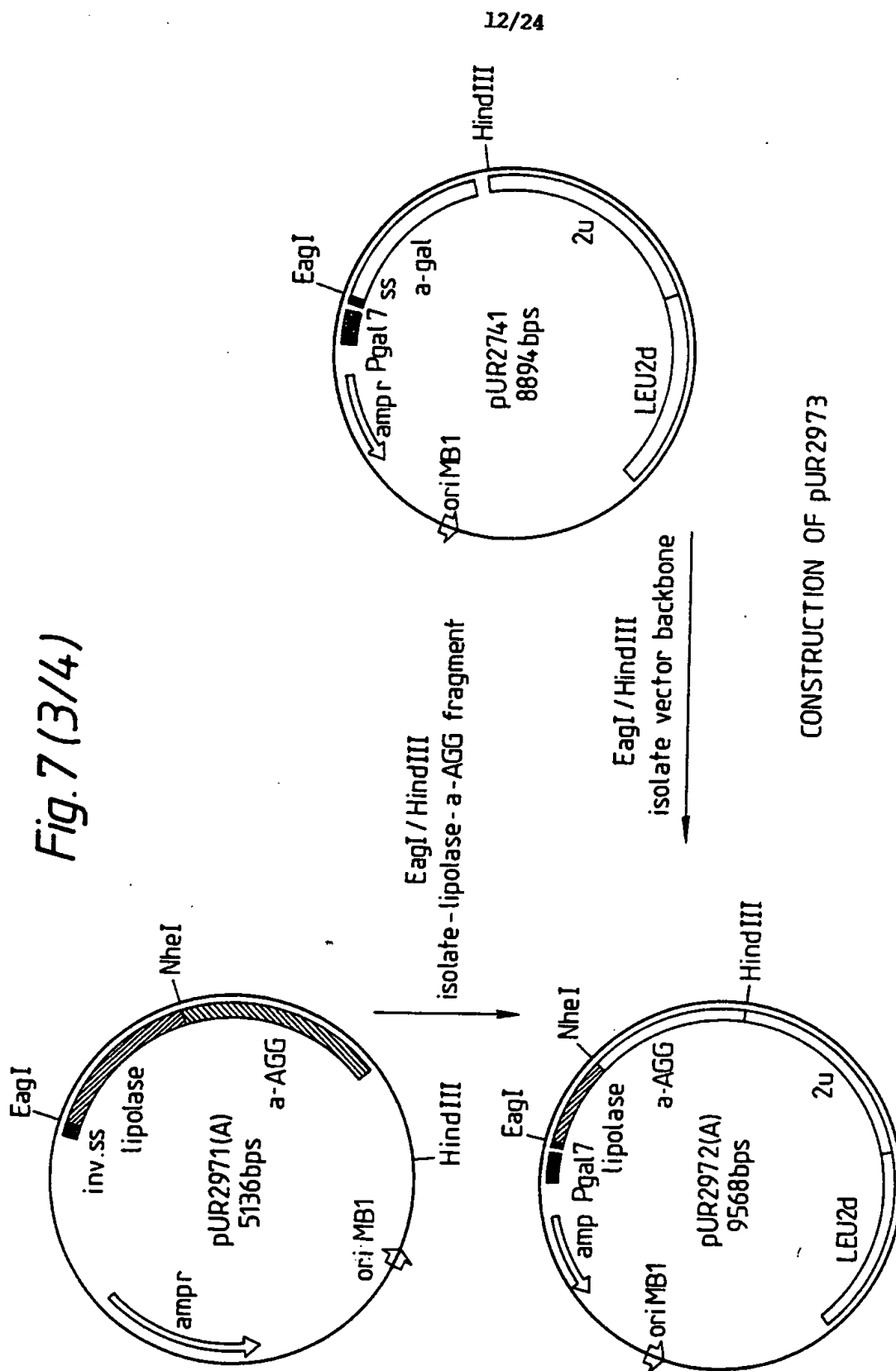


Fig. 7 (3/4)



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Fig. 7 (4/4)

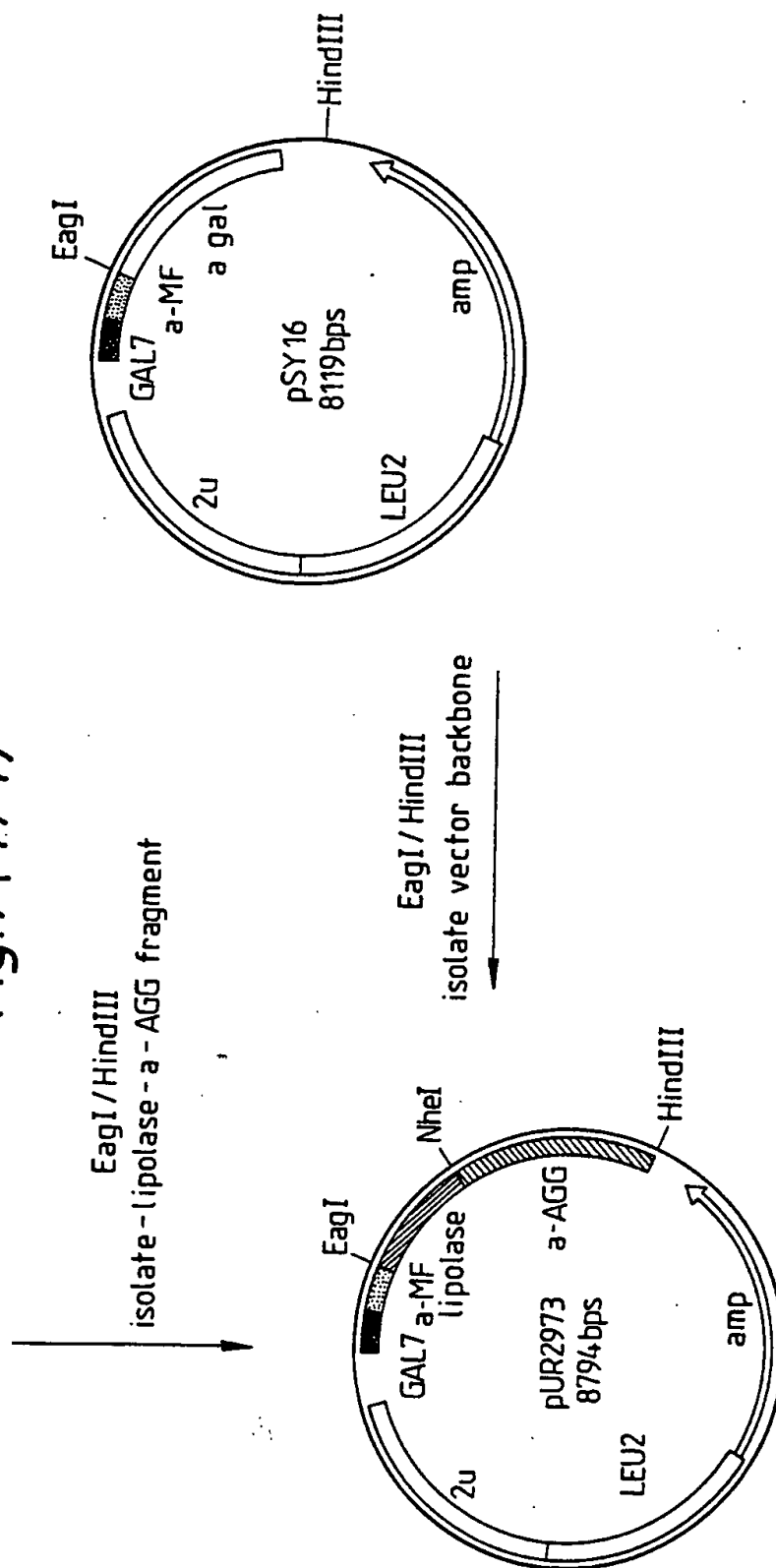


FIGURE 8, 1/2

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DNA SEQUENCE OF LIPASE B:

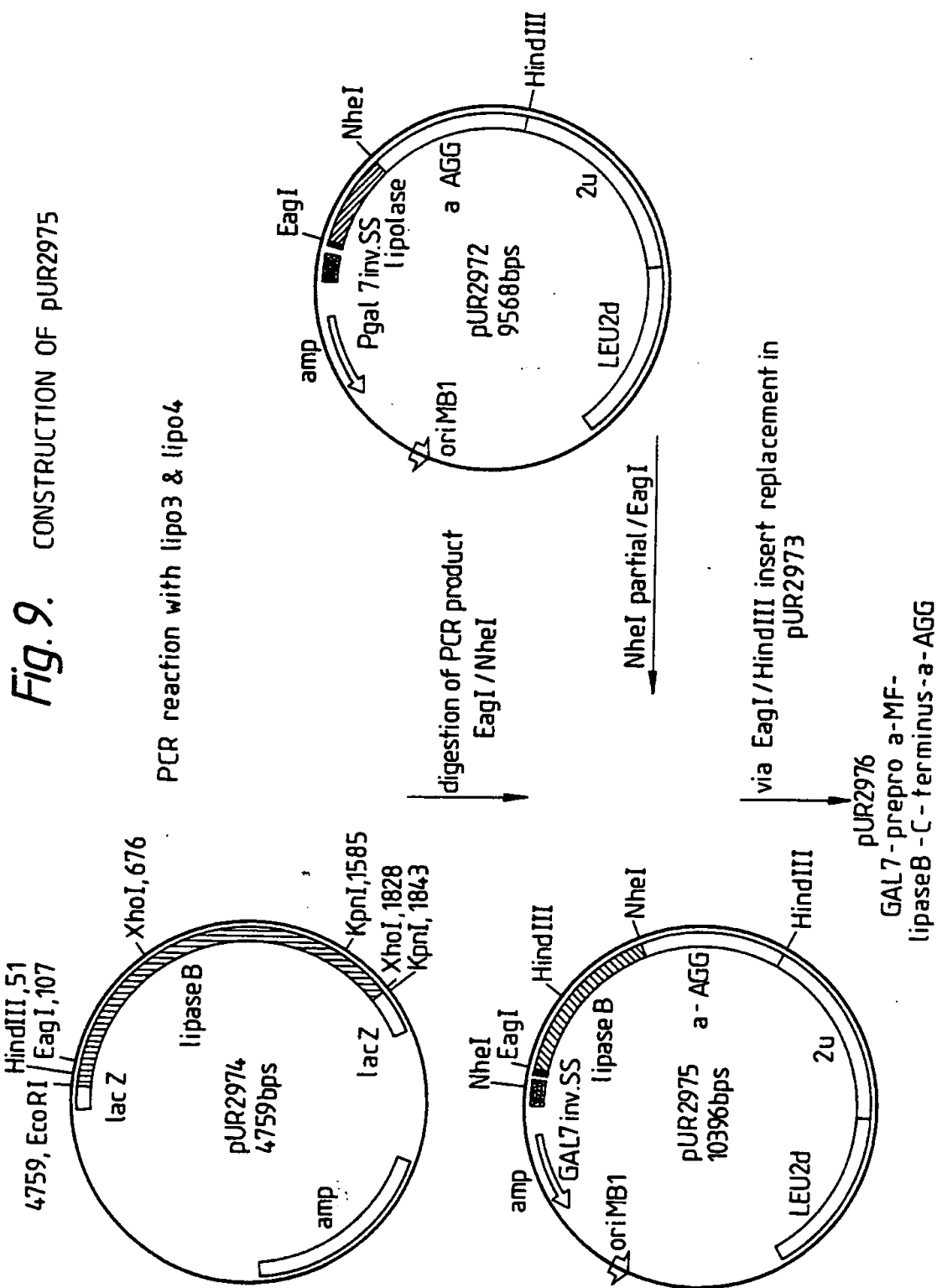
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1  AATTCGGCAC GAGATTCCTT TGATTTGCAA CTGTTAATCA
41 TGGTTTCCAA AAGCTTTTTT TTGGCTGCGG CGCTCAACGT
81 AGTGGGCACC TTGGCCCAGG CCCCCACGGC CGTTCTTAAT
121 GGCAACGAGG TCATCTCTGG TGTCCTTGAG GGCAAGGTTG
161 ATACCTTCAA GGAATCCCA TTTGCTGACC CTCCTGTTGG
201 TGACTTGCGG TTCAAGCACC CCCAGCCTTT CACTGGATCC
241 TACCAGGGTC TTAAGGCCAA CGACTTCAGC TCTGCTTGTA
281 TGCAGCTTGA TCCTGGCAAT GCCTTTTCTT TGCTTGACAA
321 AGTAGTGGGC TTGGGAAAGA TTCTTCCTGA TAACCTTAGA
361 GGCCCTCTTT ATGACATGGC CCAGGGTAGT GTCTCCATGA
401 ATGAGGACTG TCTCTACCTT AACGTTTTCC GCCCGCTGG
441 CACCAAGCCT GATGCTAAGC TCCCCGTCAT GGTTTGGATT
481 TACGGTGGTG CCTTTGTGTT TGGTTCTTCT GCTTCTTACC
521 CTGGTAACGG CTACGTCAAG GAGAGTGTGG AAATGGGCCA
561 GCCTGTTGTG TTTGTTTCCA TCAACTACCG TACCGGCCCC
601 TATGGATTCT TGGGTGGTGA TGCCATCACC GCTGAGGGCA
641 ACACCAACGC TGGTCTGCAC GACCAGCGCA AGGGTCTCGA
681 GTGGGTTAGC GACAACATTG CCAACTTTGG TGGTGATCCC
721 GACAAGGTCA TGATTTTCGG TGAGTCCGCT GGTGCCATGA
761 GTGTTGCTCA CCAGCTTGTT GCCTACGGTG GTGACAACAC
801 CTACAACGGA AAGCAGCTTT TCCACTCTGC CATTCTTCAG
841 TCTGGCGGTC CTCTTCCTTA CTTTGACTCT ACTTCTGTTG
881 GTCCCGAGAG TGCCTACAGC AGATTTGCTC AGTATGCCGG
921 ATGTGACACC AGTGCCAGTG ATAATGACAC TCTGGCTTGT
961 CTCCGCAGCA AGTCCAGCGA TGTCTTGCA CAGTGCAGCA
1001 ACTCGTATGA TCTTAAGGAC CTGTTTGGTC TGCTCCCTCA
1041 ATTCTTTGGA TTTGGTCCCA GACCCGACGG CAACATTATT
1081 CCCGATGCCG CTTATGAGCT CTACCGCAGC GGTAGATACG
1121 CCAAGGTTCC CTACATTACT GGCAACCAGG AGGATGAGGG
1161 TACTATTCTT GCCCCGTTG CTATTAATGC TACCACTACT
1201 CCCCATGTGA AGAAGTGGTT GAAGTACATT TGTAGCCAGG
1241 CTTCTGACGC TTCGCTTGAT CGTGTTTTGT CGCTCTACCC
1281 CGGCTCTTGG TCGGAGGGTT CACCATTCCG CACTGGTATT
1321 CTTAATGCTC TTACCCCTCA GTTCAAGCGC ATTGCTGCCA
1361 TTTTCACTGA TTTGCTGTTC CAGTCTCCTC GTCGTGTTAT
1401 GCTTAACGCT ACCAAGGACG TCAACCGCTG GACTTACCTT
1441 GCCACCCAGC TCCATAACCT CGTTCCATTT TTGGGTACTT
1481 TCCATGGCAG TGATCTTCTT TTTCAATACT ACGTGACCT
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FIGURE 8, 2/2

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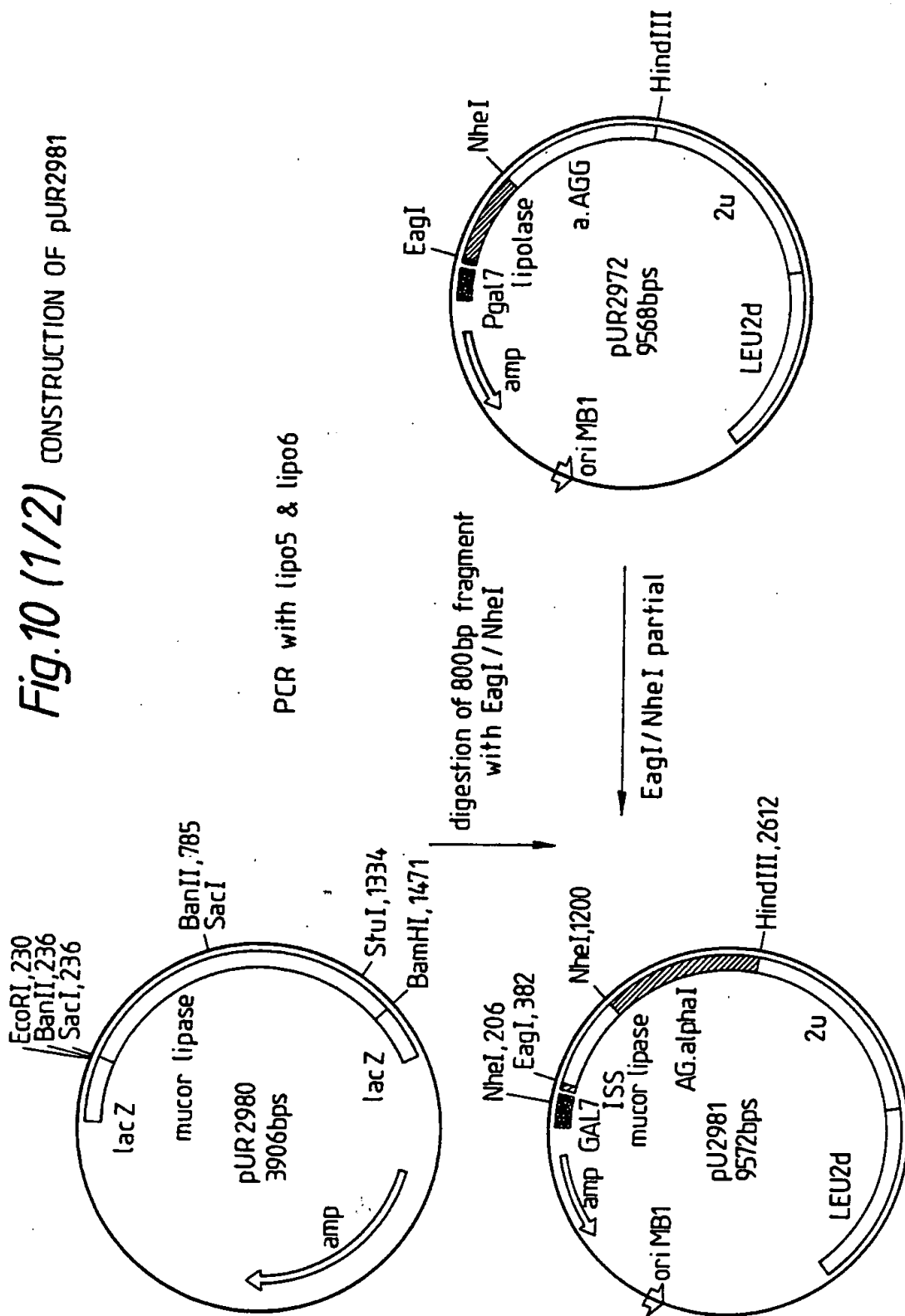
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1681 AGAATCGAGG GAATCTCGAA CTTTGAGTCT GACGTTACTC
1721 TCTTCGGTTA ATCCCATTTA GCAAGTTTGT TGTATTTCAA
1761 GTATACCAGT TGATGTAATA TATCAATAGA TTACAAATTA
1801 ATTAGTGAAA AAAAAAAAAA AAAAAAAC 1828

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Fig.10 (1/2) CONSTRUCTION OF pUR2981



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Fig.10 (2/2) CONSTRUCTION OF pUR2982

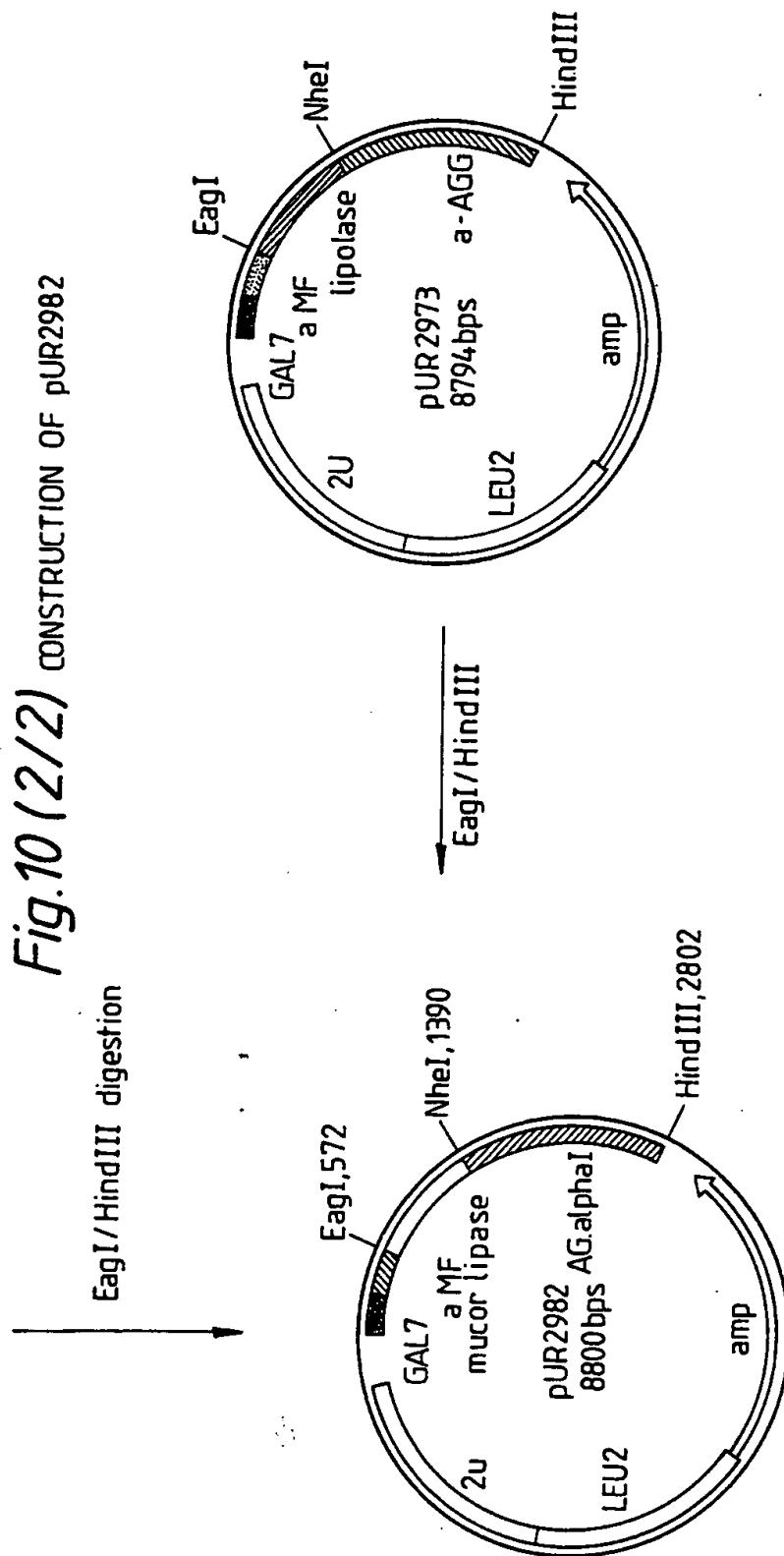


FIGURE 11, 1/2

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DNA SEQUENCE OF FLO1:

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1  ATGACAATGC CTCATCGCTA TATGTTTTTG GCAGTCTTTA
41 CACTTCTGGC ACTAACTAGT GTGGCCTCAG GAGCCACAGA
81 GCGGTGCTTA CCAGCAGGCC AGAGGAAAAG TGGGATGAAT
121 ATAAATTTTT ACCAGTATTC ATTGAAAGAT TCCTCCACAT
161 ATTCGAATGC AGCATATATG GCTTATGGAT ATGCCTCAAA
201 AACCAAATA GGTTCGTGTCG GAGGACAAAC TGATATCTCG
241 ATTGATTATA ATATTCCCTG TGTTAGTTCA TCAGGCACAT
281 TTCCTTGTC TCAAGAAGAT TCCTATGGAA ACTGGGGATG
321 CAAAGGAATG GGTGCTTGTT CTAATAGTCA AGGAATTGCA
361 TACTGGAGTA CTGATTTATT TGGTTTCTAT ACTACCCCAA
401 CAAACGTAAC CCTAGAAATG ACAGGTTATT TTTTACCACC
441 ACAGACGGGT TCTTACACAT TCAAGTTTGC TACAGTTGAC
481 GACTCTGCAA TTCTATCAGT AGGTGGTGCA ACCGCGTTCA
521 ACTGTTGTGC TCAACAGCAA CCGCCGATCA CATCAACGAA
561 CTTTACCATT GACGGTATCA AGCCATGGGG TGGAAGTTTG
601 CCACCTAATA TCGAAGGAAC CGTCTATATG TACGCTGGCT
641 ACTATTATCC AATGAAGGTT GTTTACTCGA ACGCTGTTTC
681 TTGGGGTACA CTTCCAATTA GTGTGACACT TCCAGATGGT
721 ACCACTGTAA GTGATGACTT CGAAGGGTAC GTCTATTCCT
761 TTGACGATGA CCTAAGTCAA TCTAACTGTA CTGTCCCTGA
801 CCCTTCAAAT TATGCTGTCA GTACCACTAC AACTACAACG
841 GAACCATGGA CCGGTACTTT CACTTCTACA TCTACTGAAA
881 TGACCACCGT CACCGGTACC AACGGCGTTC CAACTGACGA
921 AACCGTCATT GTCATCAGAA CTCCAACCAG TGAAGGTCTA
961 ATCAGCACCA CCACTGAACC ATGGACTGGC ACTTTCCTT
1001 CGACTTCCAC TGAGGTTACC ACCATCACTG GAACCAACGG
1041 TCAACCAACT GACGAAACTG TGATTGTTAT CAGAACTCCA
1081 ACCAGTGAAG GTCTAATCAG CACCACCACT GAACCATGGA
1121 CTGGTACTTT CACTTCTACA TCTACTGAAA TGACCACCGT
1161 CACCGGTACT AACGGTCAAC CAACTGACGA AACCGTGATT
1201 GTTATCAGAA CTCCAACCAG TGAAGGTTTG GTTACAACCA
1241 CCACTGAACC ATGGACTGGT ACTTTTACTT CGACTTCCAC
1281 TGAAATGTCT ACTGTCCTG GAACCAATGG CTTGCCAACT
1321 GATGAAACTG TCATTGTTGT CAAAACTCCA ACTACTGCCA
1361 TCTCATCCAG TTTGTCATCA TCATCTTCAG GACAAATCAC
1401 CAGCTCTATC ACGTCTTCGC GTCCAATTAT TACCCCATTC
1441 TATCCTAGCA ATGGAAC TTC TGTGATTCT TCCTCAGTAA
1481 TTTCTTCCTC AGTCACTTCT TCTCTATTCA CTTCTTCTCC
1521 AGTCATTCTC TCCTCAGTCA TTTCTTCTTC TACAACAACC
1561 TCCACTTCTA TATTTTCTGA ATCATCTAAA TCATCCGTCA
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FIGURE 11, 2/2

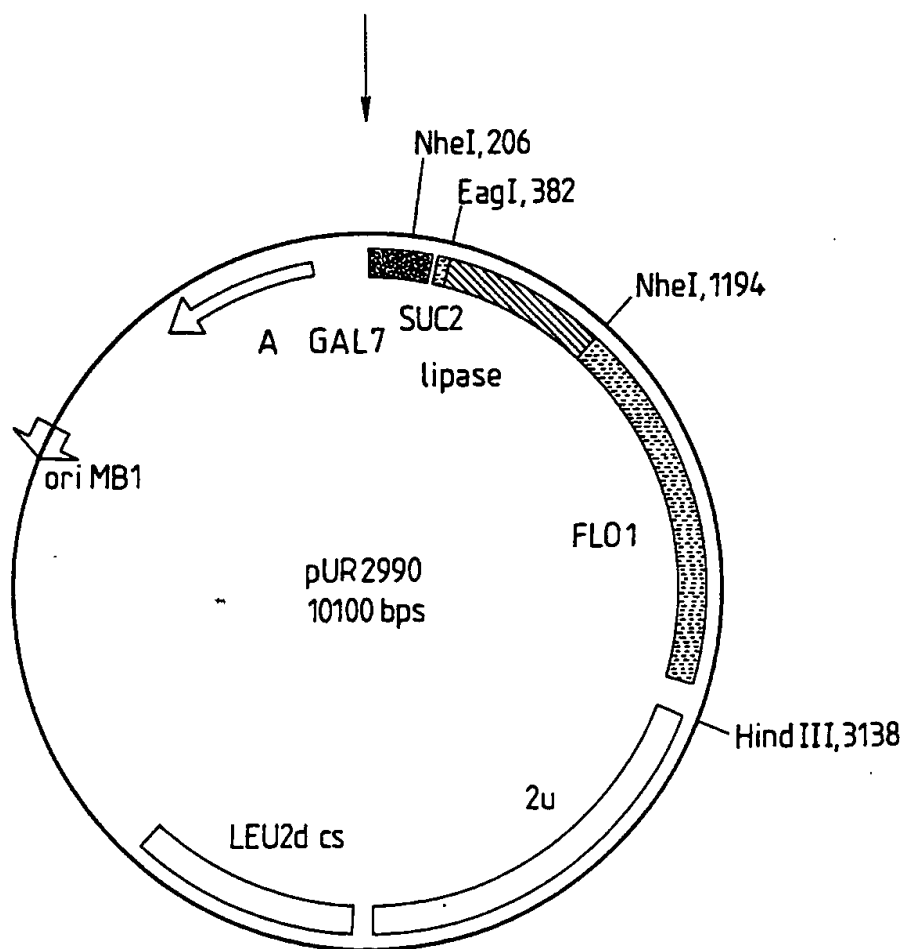
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1681	TCTTCTGAAT	CATCAAAATC	TCCTACATAT	TCTTCTTCAT
1721	CATTACCACT	TGTTACCAGT	GCGACAACAA	GCCAGGAAAC
1761	TGCTTCTTCA	TTACCACCTG	CTACCACTAC	AAAAACGAGC
1801	GAACAAACCA	CTTTGGTTAC	CGTGACATCC	TGCGAGTCTC
1841	ATGTGTGCAC	TGAATCCATC	TCCCCTGCGA	TTGTTTCCAC
1881	AGCTACTGTT	ACTGTTAGCG	GCGTCACAAC	AGAGTATACC
1921	ACATGGTGCC	CTATTTCTAC	TACAGAGACA	ACAAAGCAAA
1961	CCAAAGGGAC	AACAGAGCAA	ACCACAGAAA	CAACAAAACA
2001	AACCACGGTA	GTTACAATTT	CTTCTTGTGA	ATCTGACGTA
2041	TGCTCTAAGA	CTGCTTCTCC	AGCCATTGTA	TCTACAAGCA
2081	CTGCTACTAT	TAACGGCGTT	ACTACAGAAT	ACACAACATG
2121	GTGTCCTATT	TCCACCACAG	AATCGAGGCA	ACAAACAACG
2161	CTAGTTACTG	TTACTTCCTG	CGAATCTGGT	GTGTGTTCCG
2201	AAACTGCTTC	ACCTGCCATT	GTTTCGACGG	CCACGGCTAC
2241	TGTGAATGAT	GTTGTTACGG	TCTATCCTAC	ATGGAGGCCA
2281	CAGACTGCGA	ATGAAGAGTC	TGTCAGCTCT	AAAATGAACA
2321	GTGCTACCGG	TGAGACAACA	ACCAATACTT	TAGCTGCTGA
2361	AACGACTACC	AATACTGTAG	CTGCTGAGAC	GATTACCAAT
2401	ACTGGAGCTG	CTGAGACGAA	AACAGTAGTC	ACCTCTTCGC
2441	TTTCAAGATC	TAATCACGCT	GAAACACAGA	CGGCTTCCGC
2481	GACCGATGTG	ATTGGTCACA	GCAGTAGTGT	TGTTTCTGTA
2521	TCCGAAACTG	GCAACACCAA	GAGTCTAACA	AGTTCCGGGT
2561	TGAGTACTAT	GTCGCAACAG	CCTCGTAGCA	CACCAGCAAG
2601	CAGCATGGTA	GGATATAGTA	CAGCTTCTTT	AGAAATTTCA
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2681	TTTAA	2685-		

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Fig. 12.

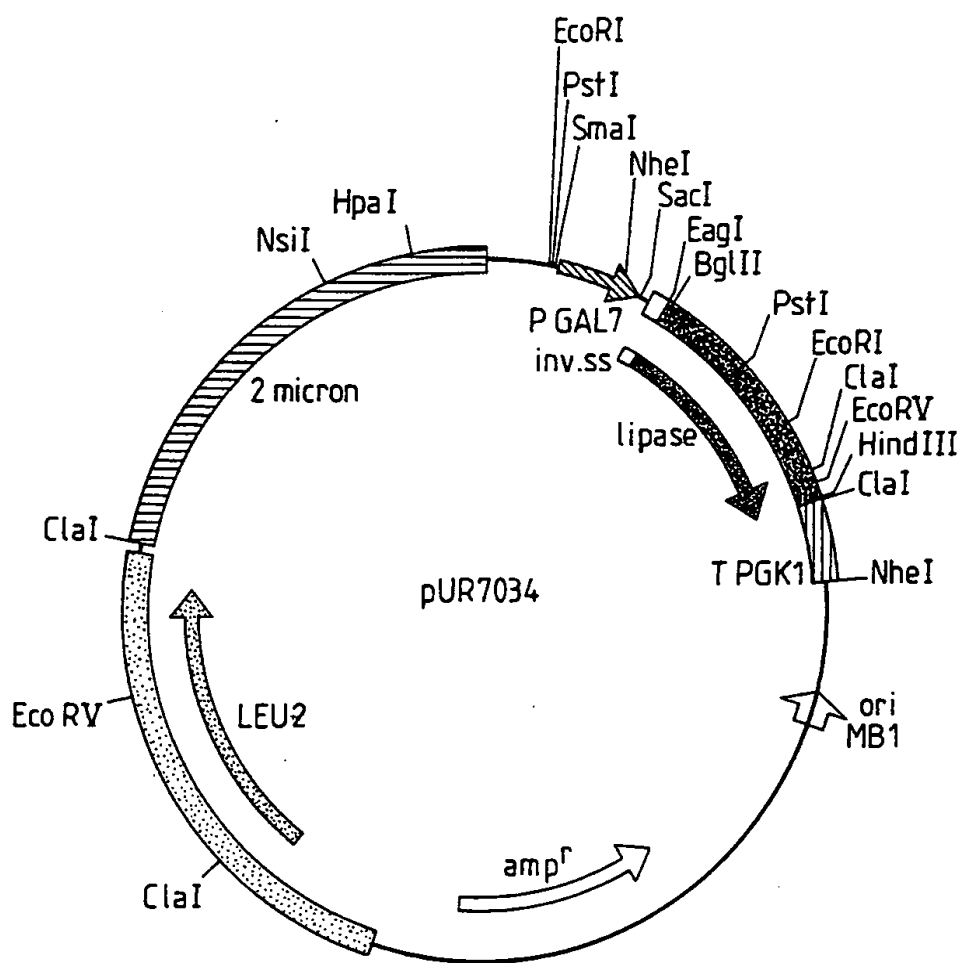
CONSTRUCTION OF pUR2990

PCR with oligonucleotides pcrflo1 & pcrflo2
Isolate 1950 bp fragment
cut with NheI and HindIII
ligate into HindIII/ NheI (p) digested pUR2972



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Fig. 13.



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Fig.14.

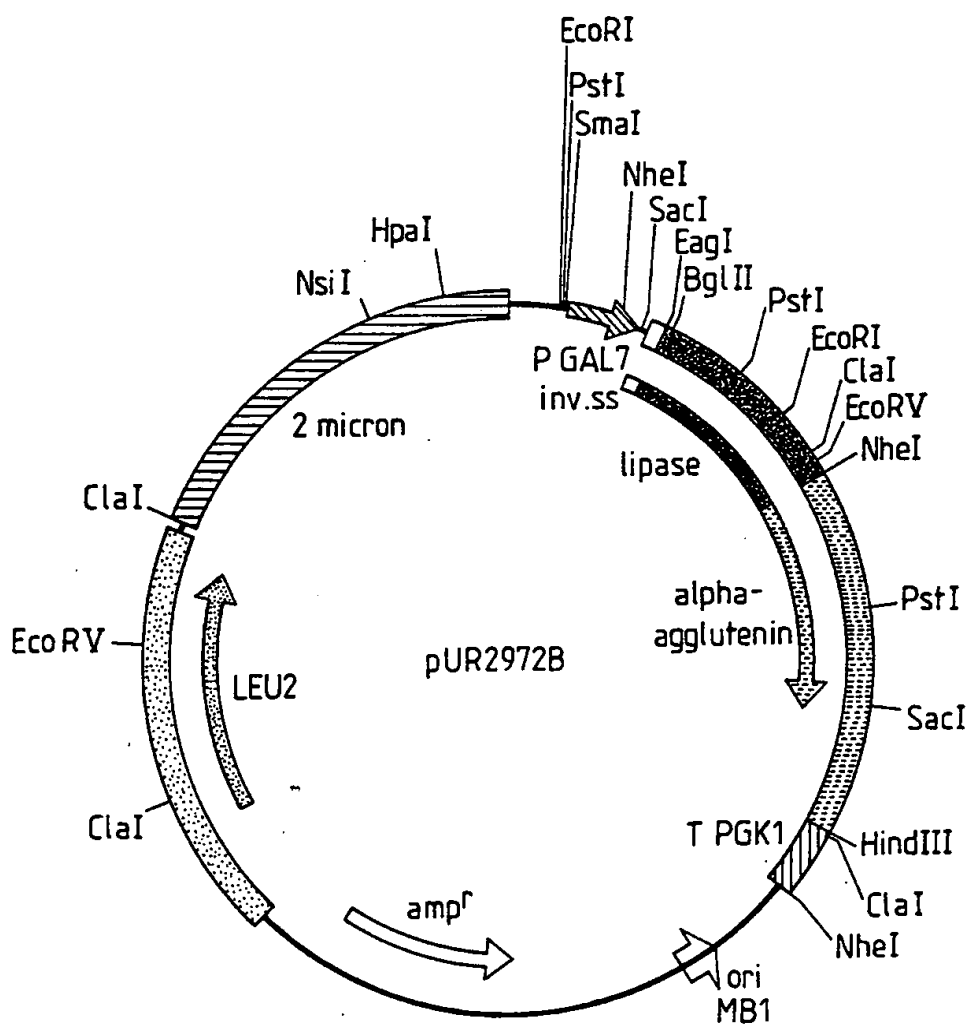
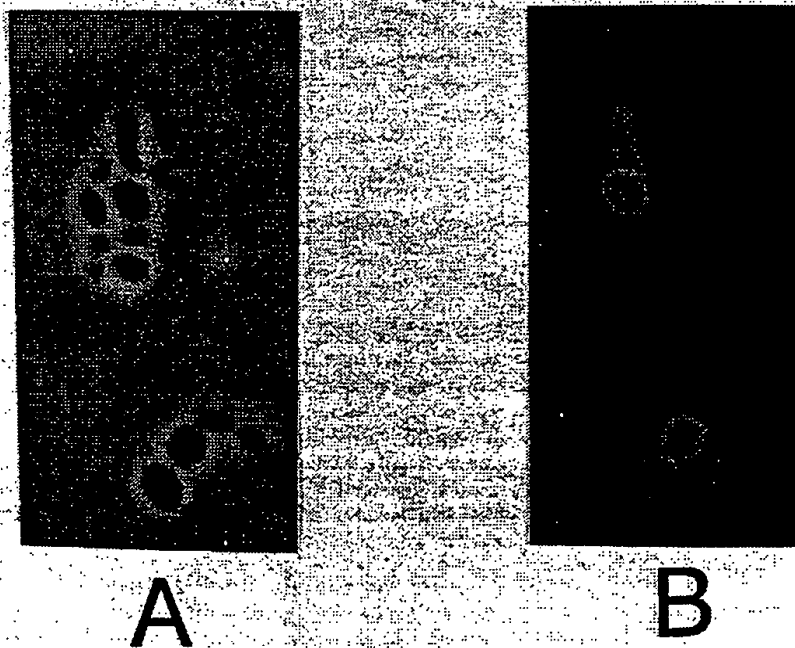


Fig.15.



(1/2)

(2/2)

INTERNATIONAL SEARCH REPORT

International Application No

PCT/EP 93/01763

I. CLASSIFICATION OF SUBJECT MATTER (If several classification symbols apply, indicate all) ⁶		
According to International Patent Classification (IPC) or to both National Classification and IPC Int.Cl. 5 C12N15/62; C12N15/56; C12N15/55; C12N15/53 C12N1/19; C12N11/16; //(C12N1/19, C12R1:865)		
II. FIELDS SEARCHED		
Minimum Documentation Searched ⁷		
Classification System	Classification Symbols	
Int.Cl. 5	C12N	
Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched ⁸		
III. DOCUMENTS CONSIDERED TO BE RELEVANT⁹		
Category ¹⁰	Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹²	Relevant to Claim No. ¹³
X	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA. vol. 89, April 1992, WASHINGTON US pages 2713 - 2717 JOSEPH A. FRANCISCO ET AL 'Transport and anchoring of beta-lactamase to the external surface of Escherichia coli' see the whole document ---	1-4, 7-8, 10, 12-13, 15-16, 18
X	JOURNAL OF BACTERIOLOGY vol. 171, no. 9, September 1989, pages 4569 - 4576 GEORGE ET AL 'The hydrophobic domain of cytochrome b5 is capable of anchoring beta-galactosidase in Escherichia coli membranes' * the whole article especially page 4569 lines 6-20 * --- <div style="text-align: right;">-/--</div>	1-4, 12-13, 16, 18
<div style="display: flex; justify-content: space-between;"> <div style="width: 45%;"> <p>¹⁰ Special categories of cited documents:</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> </div> <div style="width: 45%;"> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"A" document member of the same patent family</p> </div> </div>		
IV. CERTIFICATION		
Date of the Actual Completion of the International Search <div style="text-align: center; font-weight: bold;">28 SEPTEMBER 1993</div>		Date of Mailing of this International Search Report <div style="text-align: center; font-weight: bold;">01-10-1993</div>
International Searching Authority <div style="text-align: center; font-weight: bold;">EUR PEAN PATENT OFFICE</div>		Signature of Authorized Officer <div style="text-align: center; font-weight: bold;">LE CORNEC N.D.R.</div>

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
X	JOURNAL OF CELL BIOLOGY vol. 107, September 1988, pages 865 - 876 SCOTT W. HIEBERT ET AL 'Cell surface expression of glycosylated, nonglycosylated, and truncated forms of a cytoplasmic protein pyruvate kinase' see the whole document	1-4
X	WO,A,8 907 140 (COMMONWEALTH SCIENTIFIC AND INDUSTRIAL RESEARCH ORGANISATION) 10 August 1989 see page 5, line 30 - page 6, line 22 see page 12, line 13 - line 19	1-4
A	JOURNAL OF BIOLOGICAL CHEMISTRY. vol. 265, no. 6, 25 February 1990, BALTIMORE US pages 3161 - 3167 CECIL C. CHEN ET AL 'Complete nucleotide sequence of the Streptococcal C5a peptidase of Streptococcus pyogenes' see the whole document	
A	CHEMICAL ABSTRACTS, vol. 102, no. 3, 21 January 1985, Columbus, Ohio, US; abstract no. 18728c, EMR, SCOTT D. ET AL 'Invertase beta-galactosidase hybrid proteins fail to be transported from the endoplasmic reticulum in Saccharomyces cerevisiae' page 176 ;column L ; see abstract	
A	& MOLECULAR AND CELLULAR BIOLOGY vol. 4, no. 11, 1984, WASHINGTON US pages 2347 - 2355	1,3
A	MOLECULAR AND CELLULAR BIOLOGY vol. 8, no. 4, April 1988, WASHINGTON US pages 1709 - 1714 S. VIJAYA ET AL 'Transport to the cell surface of a peptide sequence attached to the truncated C terminus of an N-terminally anchored integral membrane protein' see the whole document	1,3

EP 9301763
SA 76719

28/09/93

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A-8907140	10-08-89	AU-A- 3045389	25-08-89
		EP-A- 0398944	28-11-90



US006027910A

United States Patent [19]
Klis et al.

[11] **Patent Number:** **6,027,910**
 [45] **Date of Patent:** **Feb. 22, 2000**

[54] **PROCESS FOR IMMOBILIZING ENZYMES
 TO THE CELL WALL OF A MICROBIAL
 CELL BY PRODUCING A FUSION PROTEIN**

[75] **Inventors:** Franciscus M Klis, Amsterdam;
 Maarten P Schreuder, Diemen, both of
 Netherlands; Holger Y Toschka, Reken,
 Germany; Cornelis T Verrips,
 Maassluis, Netherlands

[73] **Assignee:** Unilever Patent Holdings B.V.,
 Vlaardingen, Netherlands

[21] **Appl. No.:** **08/362,525**

[22] **PCT Filed:** **Jul. 7, 1993**

[86] **PCT No.:** **PCT/EP93/01763**

§ 371 Date: **Feb. 27, 1995**

§ 102(e) Date: **Feb. 27, 1995**

[87] **PCT Pub. No.:** **WO94/01567**

PCT Pub. Date: **Jan. 20, 1994**

[30] **Foreign Application Priority Data**

Jul. 8, 1992 [EP] European Pat. Off. 92202080
 Dec. 14, 1992 [EP] European Pat. Off. 92203899

[51] **Int. Cl.⁷** **C12N 15/00; C12N 1/20;**
C12P 1/00

[52] **U.S. Cl.** **435/41; 435/69.7; 435/69.8;**
435/69.9; 435/172.1; 435/172.3; 435/252.3;
435/320.1

[58] **Field of Search** **435/69.7, 69.8,**
435/71.1, 71.2, 172.1, 252.1, 252.3, 252.33,
320.1, 183, 69.9, 189, 195, 198, 41, 172.3

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Primary Examiner—Eric Grimes

Assistant Examiner—Nashaat T. Nashed

Attorney, Agent, or Firm—Cushman Darby & Cushman IP Group of Pillsbury Madison & Sutro

[57] **ABSTRACT**

A method is provided for immobilizing an enzyme, comprising immobilizing the enzyme or a functional part thereof to the cell wall of a microbial cell using recombinant DNA techniques. The enzyme is immobilized by linking it to the C-terminal part of a protein that ensures anchoring in the cell wall. Also provided is a recombinant polynucleotide comprising a structural gene encoding an enzyme protein, a part of a gene encoding the C-terminal part of a protein capable of anchoring in a eukaryotic or prokaryotic cell wall, as well as a signal sequence, in addition to a chimeric protein encoded by the recombinant polynucleotide and a vector and a microorganism containing the polynucleotide. The microorganism is suitable for carrying out enzymatic processes on an industrial scale.

17 Claims, 24 Drawing Sheets

FIG. 1A

1	AAGCTTTAGG	TAAGGGAGGC	AGGGGGAAAA	GATACTGAAA
41	TGACGGAAAA	CGAGAATATG	GAGCAGGGAG	CAACTTTTAG
81	AGCTTTACCC	GTTAAAAGGT	CAAATCGAGG	CTTCCTGCCT
121	TTGTCTGATT	TTAGTAGTAC	CGGAAGGTTT	ATTACGCCCA
161	AGAACAGTGC	TTGAATTGAG	TTCTCGGGAC	ACGGGAAAGA
201	CAATGGAAGA	AAAATTTACA	TTCAGTAGCC	TTATATATGA
241	AATGCTGCCA	AGCCACGTCT	TTATAAGTAG	ATAATGTCCC
281	ATGAGCTGAA	CTATGGGAAT	TTATGACGCA	GTTCAATTGA
321	TATATATTAC	ATTAACCTCT	TAGTTTAAAC	TCTGAATTGT
361	TTTATAAAAT	AACTTTTTGA	ATTTTTTTTAT	GATCGCTTAG
401	TTAAGTCTAT	TATATCAGGT	TTTTTTCATTC	ATCATAATTG
441	TTCGTTAAAT	ATGAGTATAT	TTAAATACAG	GAATTAGTAT
481	CATTTGCAGT	CACGAAAAGG	GCCGTTTCAT	AGAGAGTTTT
521	CTTAATAAAG	TTGAGGGTTT	CCGTGATAGT	TTTGAGGGGT
561	TGTTTGAAC	AGATTTACGC	TTACCTTTCA	ACTGATTAAT
601	TTTTTCAGCG	GGCTTATCAT	AATCATCCAT	CATAGCAGTC
641	TTTCTGGACT	TCGTGAGGA	CTGGCTTTCT	GAATTTTGAC
681	GGTCCCTATT	AGCTCCAGTT	GGAGGAATTG	AGTTACCTAC
721	AACTGGCAAG	AGGTCTTTGT	TTGGATTCAA	AATAGGACTT
761	TGTGGTAGCA	GTTTGGTTTT	ATTCAATCTA	AAGATATGAG
801	AAACAGGTTT	TAAGTAAATC	GATACTATTG	TACCAATGTT
841	TAGCTCCAAT	TCCTCCAAAA	CGGTGGGATC	TAATTTTGTG
881	TTCAATTCTA	TTAGTGGCAA	CTCTCCGTCC	AGTACTGATT
921	TTAAAGATT	AAAAGTTATC	GCGTTTGATA	TACGAGACGT
961	TTTCGTTAAT	GACAGCAATC	TCCAATACAT	CAGTGTTTTA
1001	TCTCTTAAGT	CAGGATTATT	TTCTGATCG	GTGCATCCTT
1041	TTAATAAATC	CATACAAAGT	TCTTCAGTTT	CCTTTGTAGG
1081	ATTTCTGATG	AAGAATTTTA	TTGCTGAGTT	CAGAATGGAA
1121	AATTGCACTT	CTAGCGTCTC	ATTAAACATG	TTTGAGGAAA
1161	AAACTCTAAA	TAACTCCAGG	TAGTTTGGAA	TTACATCCGA
1201	ATATTGCGTT	ATTATCCAGA	TCATAGCGTT	TTTTGATTCA
1241	GGTTCCTGTA	CAACTTCAGT	GTGTTTGACT	AGTTCTGTTA
1281	CGTTTGCTTT	AAAATTATTG	GGATATTTCC	TCAAAATATT
1321	TCTGAAAACC	GAAATAATCT	CCTGGACGAC	ATAATCAACA
1361	CCGAATTCTA	ACAAATCTAG	TAGCACAGCG	ACACAATCGT
1401	GTACAGAGTC	TTCATCTAGC	TTAACAGCGA	GATTACCAAT
1441	GGCTCTGACT	GATTTCTTG	ACATTTGAAT	ATCAATATCT
1481	GTAGCATATT	GTTCCAACCT	TTCTAGAATT	CTTGGTAATG
1521	TTTCCTTGTT	AGCTAAAAGA	TATAAACACT	CTAATTTCTG
1561	GTCTTTGATG	TATATGGGGT	CATTGTACTC	GATGAAAAAA

FIG. 1B

1601	TACGAAATGT	CTAGCCTGAG	TAGAGATGAC	TCCCTACTCA
1641	ATAAAAGAAG	AATAACGTTT	CTTAATACTA	AAAATTGTAA
1681	TTCAGGCGGC	TTATCTAACA	AAGCTATTAC	AGAGTTAGAT
1721	AGCTTTTTCGG	CTAGAGTTTC	TTTGATGACG	TCAACATAAT
1761	TCAACAAGTA	CATGATGAAT	TTTAAAGAGT	TCAACACTAC
1801	GTATGTGTTT	ACTTGTTGCA	GGTACGGTAA	AGCTAGTTCTG
1841	ATCATTTTCAT	GGGTATCCAA	ATAATGCTGC	GGCACAACCG
1881	AAGTCGTCAA	AACTTCCAAA	ACAGTAGCCT	TATTCCACTC
1921	ATTTAATTCTG	GGTAAAAGTT	CTAGCATGTC	AAAAGCGAGT
1961	TCCAAGGGAA	TCCTGAAGGT	TCCATGTTAG	CGTTTTTTTTC
2001	GTGAATGGAA	TATAAAGTAT	GTAATGCAGC	TACAATGACT
2041	TCTGGAGAGC	TCGACTGTGC	CTTTACAATG	TCATGTAGAA
2081	TGCTTGATAA	CCCCAATACC	CTTTCATGAT	CAATTTCATC
2121	TAAATCCAAC	AGTGCGTAAA	TTGCTGTCCT	CGTCACTTGT
2161	TCAGGTGGAG	ACTTGTGATT	TACCAATGAA	ATGATACAGT
2201	CGAAGGCCTG	ATCAGATAGC	TCTTTCACCG	GGACTAATAC
2241	CAGAGTTCTT	AGTGCCATTA	TTTGTAACCT	TTCATCTCTG
2281	CTTTTGAAAT	CGTCCATTAT	AAATGGCAAA	GCCTCTCTGG
2321	CCTGCTGAGG	TTTTAATGCG	CCGATCACCC	TAATATACTC
2361	ATGGCAAATT	CTTTTCACTT	CTAGATCATC	TTCAATTTGC
2401	CAAAATTTCA	AGAGCTCAGA	AAACAGAAGG	GACATTTTCGC
2441	CATAGTTTCC	TAGAACCAAA	TTGGCGATAA	TTTTTCTCAG
2481	AGCATTTTTTC	CTTCTTGTTA	TATTCGATTT	AAACTTTTTT
2521	ACTCCAAAAT	GTTGCAGATC	TGTGACGATT	TCATTTGCTT
2561	TATATCTGGC	AAAAACTTTT	TGATCGGACA	TAAGCGAAAT
2601	ACGTCCTATT	AATGAAGTGA	ATGTTCTTGC	TGTATTCCCT
2641	TCTTGTGCAG	TAGATTAATT	CTGTTTCCAG	GCTGCGATAC
2681	TTTGATAACCG	AATACTAAAA	GTTGATGATT	TGAACGATCT
2721	CCTATTTTCT	CGCACATTTT	TGGAGCGATA	CCCGGAAGAC
2761	AGAATCGCGA	TGTTAAGAAA	ATAGTTCTGA	TGGCACTAAA
2801	GAGATCATGA	TTAAGGAAAG	GTAAGTGATA	TGCATGAATG
2841	GGAATAGGCT	TTCGAACCTG	ACGATTTAGT	TCCTTATTTC
2881	TATCCATCTA	ATCCTCCAAC	TTCAATAGGC	CTTATCTAGC
2921	TCAGAGCAGT	ATTTAATTGA	GAATAGTAGC	TTAATTGAAA
2961	CCTTACTAAA	AAAGTGTATG	GTTACATAAG	ATAAGGCGTT
3001	AAGAAGAGTA	TACATATGCA	TTATTCATTA	CCAAGACCAC
3041	TATGAATAGT	AATACCATAT	TTAGCTTTTG	AAACTCATGT
3081	TTTCTATTGT	GTTGTTTCAA	ATTCCTCTGT	TAGGCTCAAT
3121	TTAGGTTAAT	TAAATTATAA	AAAAATATAA	AAAATAAAGA
3161	AAGTTTATCC	ATCGGCACCT	CAATTCAATG	GAGTAAACAG
3201	TTTCAACACT	GAGTGGTGAA	ACATTGAACA	ACTACATGCA
3241	GTTTCCCGCC	ACGAGGCAAG	TGTAGGTCCT	TTGTCCATTT

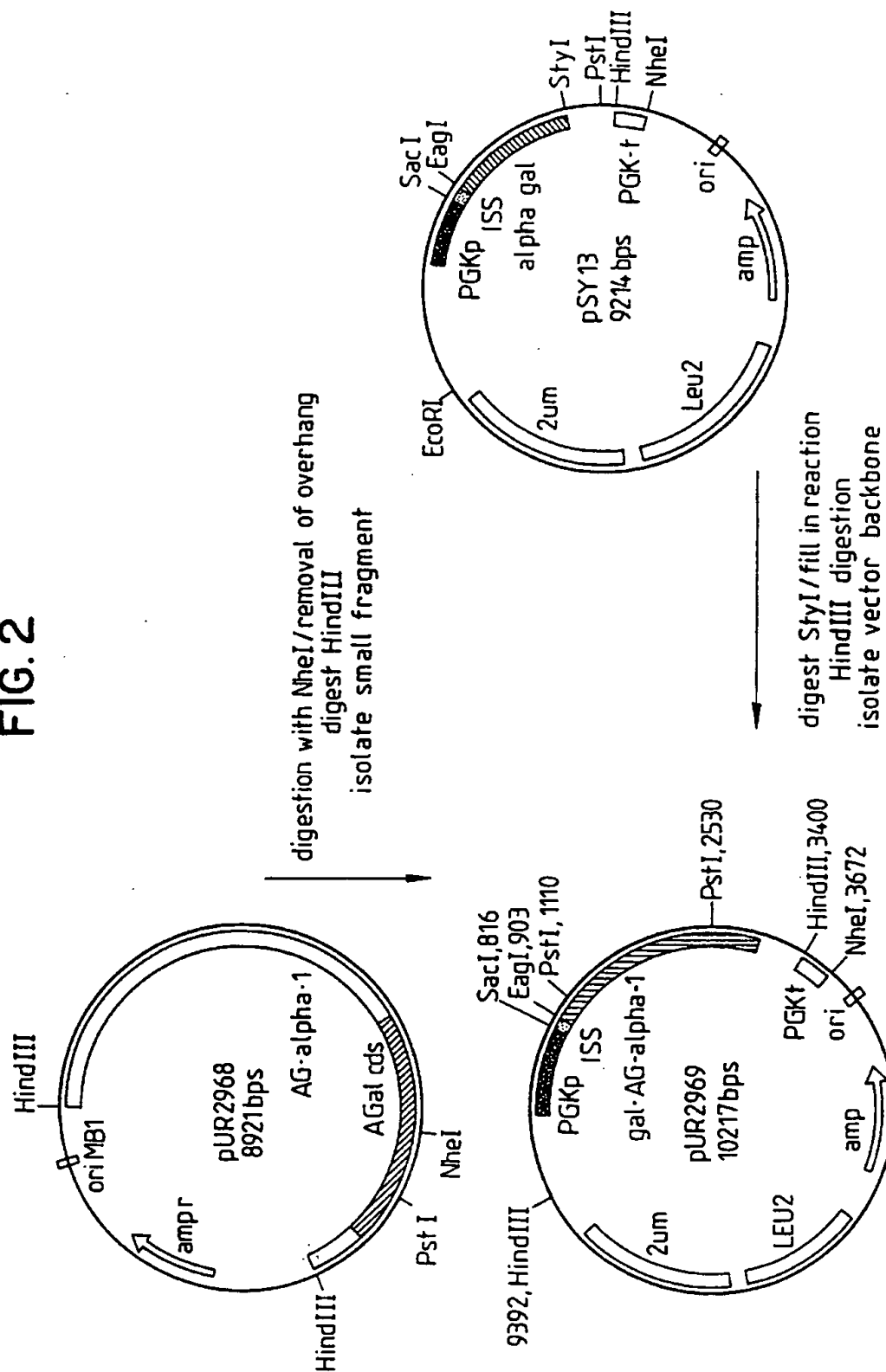
FIG. 1C

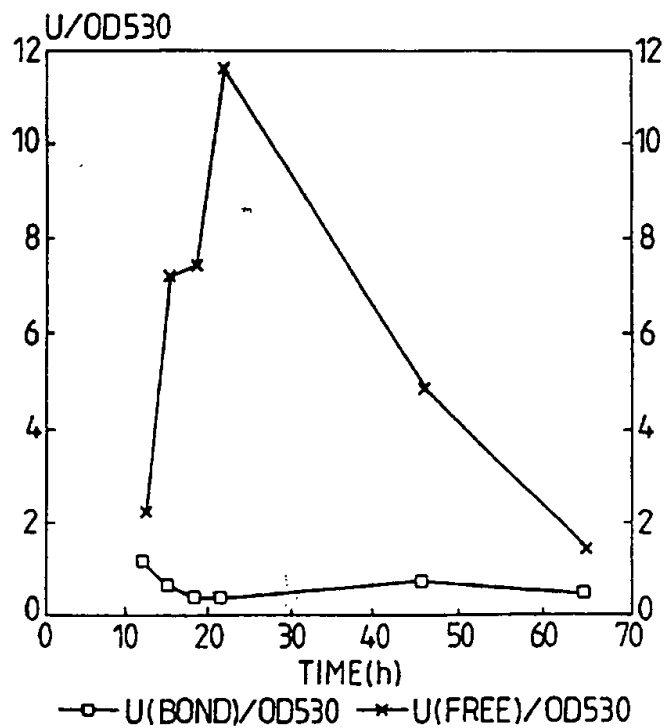
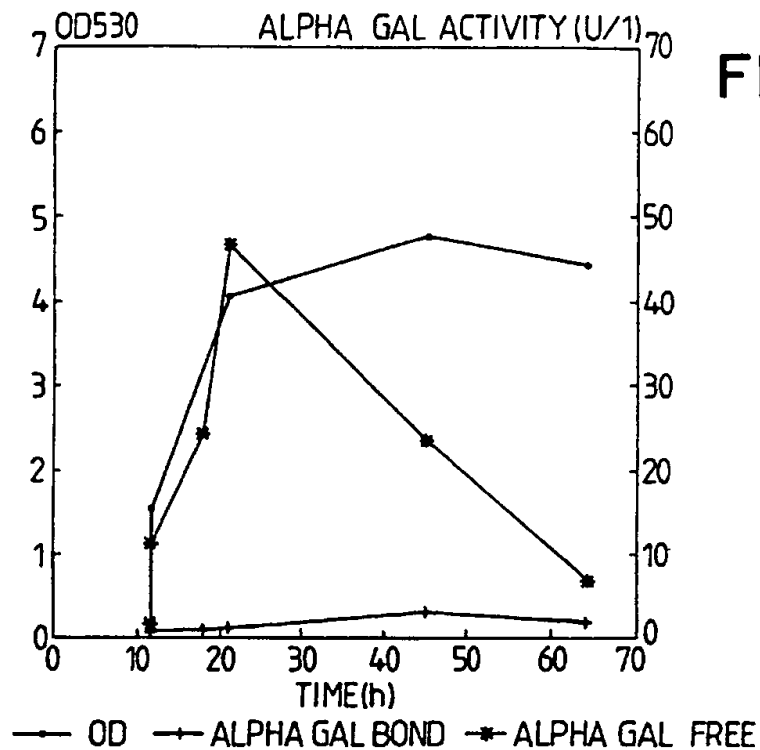
3281	CGCTTTGTTT	TGCAGGTCAT	TGATGACCTA	ATTAGGAAGG
3321	TAGAAGCCGC	TCCAGCTCAA	TAAGGAAATG	CTAAGGGTAC
3361	TCGCCTTTGG	TGTTTTACCA	TACAATGGCA	GCTTTATGTC
3401	ACTTCATTCT	TCAGTAACGG	CGCTTAAATA	TTCCCAAAAA
3441	CGTTACAATG	GAATTGTTTG	ATCATGTAAC	GAAATGCAAT
3481	CTTCTAAAAA	AAAAGCCATG	TGAATCAAAA	AAAGATTCTT
3521	TTTAGCATAC	TATAAATATG	CAAAATGCCC	TCTATTTATT
3561	CTAGTAATCG	TCCATTCTCA	TATCTTCCTT	ATATCAGTCG
3601	CCTCGCTTAA	TATAGTCAGC	ACAAAAGGAA	CAACAATTCT
3641	CCAGTTTTCA	AAATGTTTCA	TTTTCTCAAA	ATTATTCTGT
3681	GGCTTTTTTC	CTTGGCATTG	GCCTCTGCTA	TAAATATCAA
3721	CGATATCACA	TTTTCCAATT	TAGAAATTAC	TCCACTGACT
3761	GCAAATAAAC	AACCTGATCA	AGGTTGGACT	GCCACTTTTG
3801	ATTTTAGTAT	TGCAGATGCG	TCTTCCATTA	GGGAGGGCGA
3841	TGAATTCACA	TTATCAATGC	CACATGTTTA	TAGGATTAAG
3881	CTATTAAACT	CATCGCAAAC	AGCTACTATT	TCCTTAGCGG
3921	ATGGTACTGA	GGCTTTCAAA	TGCTATGTTT	CGCAACAGGC
3961	TGCATACTTG	TATGAAAATA	CTACTTTCAC	ATGTACTGCT
4001	CAAAATGACC	TGTCCTCCTA	TAATACGATT	GATGGATCCA
4041	TAACATTTTC	GCTAAATTTT	AGTGATGGTG	GTTCCAGCTA
4081	TGAATATGAG	TTAGAAAACG	CTAAGTTTTT	CAAATCTGGG
4121	CCAATGCTTG	TTAAACTTGG	TAATCAAATG	TCAGATGTGG
4161	TGAATTTTCA	TCCTGCTGCT	TTTACAGAGA	ATGTTTTTCA
4201	CTCTGGGCGT	TCAACTGGTT	ACGGTTCCTT	TGAAAGTTAT
4241	CATTTGGGTA	TGTATTGTCC	AAACGGATAT	TTCTTGGGTG
4281	GTACTGAGAA	GATTGATTAC	GACAGTTCCA	ATAACAATGT
4321	CGATTTGGAT	TGTTCTTCAG	TTTCAAGTTT	TTTCAATCAAT
4361	GATTTTAAAT	ATTGGTGGTT	CCCGCAAAGT	TACAATGATA
4401	CCAATGCTGA	CGTCACTTGT	TTTGGTAGTA	ATCTGTGGAT
4441	TACACTTGAC	GAAAAACTAT	ATGATGGGGA	AATGTTATGG
4481	GTTAATGCAT	TACAATCTCT	ACCCGCTAAT	GTAAACACAA
4521	TAGATCATGC	GTTAGAATTT	CAATACACAT	GCCTTGATAC
4561	CATAGCAAAT	ACTACGTACG	CTACGCAATT	CTCGACTACT
4601	AGGGAATTTA	TTGTTTATCA	GGGTCGGAAC	CTCGGTACAG
4641	CTAGCGCCAA	AAGCTCTTTT	ATCTCAACCA	CTACTACTGA
4681	TTTAACAAGT	ATAAACACTA	GTGCGTATTC	CACTGGATCC
4721	ATTTCCACAG	TAGAAACAGG	CAATCGAACT	ACATCAGAAG
4761	TGATCAGTCA	TGTGGTGACT	ACCAGCACAA	AACTGTCTCC
4801	AACTGCTACT	ACCAGCCTGA	CAATTGCACA	AACCAGTATC
4841	TATTCTACTG	ACTCAAATAT	CACAGTAGGA	ACAGATATTC
4881	ACACCACATC	AGAAGTGATT	AGTGATGTGG	AAACCATTAG
4921	CAGAGAAACA	GCTTCGACCG	TTGTAGCCGC	TCCAACCTCA

FIG. 1D

4961	ACAACTGGAT	GGACAGGCGC	TATGAATACT	TACATCCCGC
5001	AATTTACATC	CTCTTCTTTC	GCAACAATCA	ACAGCACACC
5041	AATAATCTCT	TCATCAGCAG	TATTTGAAAC	CTCAGATGCT
5081	TCAATTGTCA	ATGTGCACAC	TGAAAATATC	ACGAATACTG
5121	CTGCTGTTCC	ATCTGAAGAG	CCCACCTTTG	TAAATGCCAC
5161	GAGAAACTCC	TTAAATTCCT	TCTGCAGCAG	CAAACAGCCA
5201	TCCAGTCCCT	CATCTTATAC	GTCTTCCCCA	CTCGTATCGT
5241	CCCTCTCCGT	AAGCAAAACA	TTACTAAGCA	CCAGTTTTAC
5281	GCCTTCTGTG	CCAACATCTA	ATACATATAT	CAAAACGGAA
5321	AATACGGGTT	ACTTTGAGCA	CACGGCTTTG	ACAACATCTT
5361	CAGTTGGCCT	TAATTCTTTT	AGTGAAACAG	CACTCTCATC
5401	TCAGGGAACG	AAAATTGACA	CCTTTTTTAGT	GTCATCCTTG
5441	ATCGCATATC	CTTCTTCTGC	ATCAGGAAGC	CAATTGTCCG
5481	GTATCCAACA	GAATTTTACA	TCAACTTCTC	TCATGATTTT
5521	AACCTATGAA	GGTAAAGCGT	CTATATTTTT	CTCAGCTGAG
5561	CTCGGTTCGA	TCATTTTTCT	GCTTTTGTCG	TACCTGCTAT
5601	TCTAAAACGG	GTACTGTACA	GTTAGTACAT	TGAGTCGAAA
5641	TATACGAAAT	TATTGTTTAT	AATTTTCATC	CTGGCTCTTT
5681	TTTTCTTCAA	CCATAGTTAA	ATGGACAGTT	CATATCTTAA
5721	ACTCTAATAA	TACTTTTCTA	GTTCTTATCC	TTTTCCGTCT
5761	CACCGCAGAT	TTTATCATAG	TATTAAATTT	ATATTTTGTT
5801	CGTAAAAAGA	AAAATTTGTG	AGCGTTACCG	CTCGTTTCAT
5841	TACCCGAAGG	CTGTTTCAGT	AGACCACTGA	TTAAGTAAGT
5881	AGATGAAAAA	ATTTTCATCAC	CATGAAAGAG	TTCGATGAGA
5921	GCTACTTTTT	CAAATGCTTA	ACAGCTAACC	GCCATTCAAT
5961	AATGTTACGT	TCTCTTCATT	CTGCGGCTAC	GTTATCTAAC
6001	AAGAGGTTTT	ACTCTCTCAT	ATCTCATTCA	AATAGAAAGA
6041	ACATAATCAA	AAAGCTT	6057	

FIG. 2





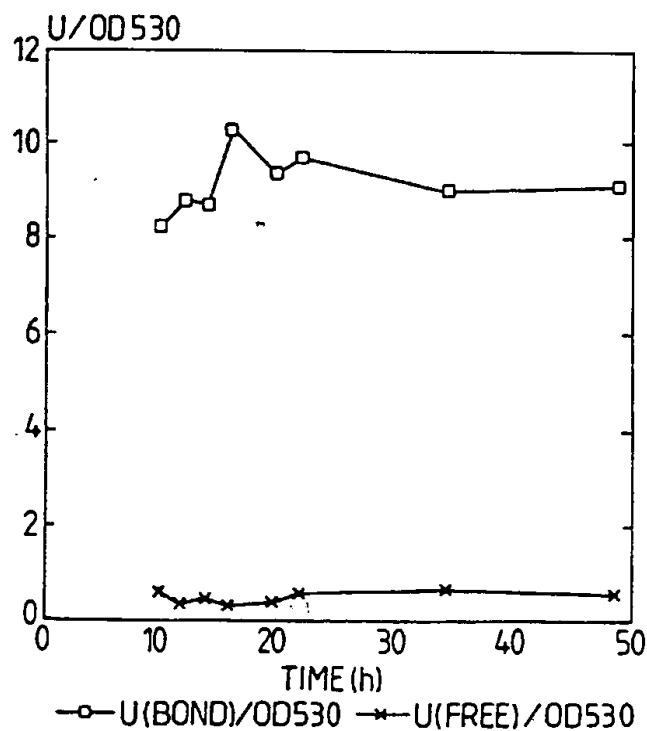
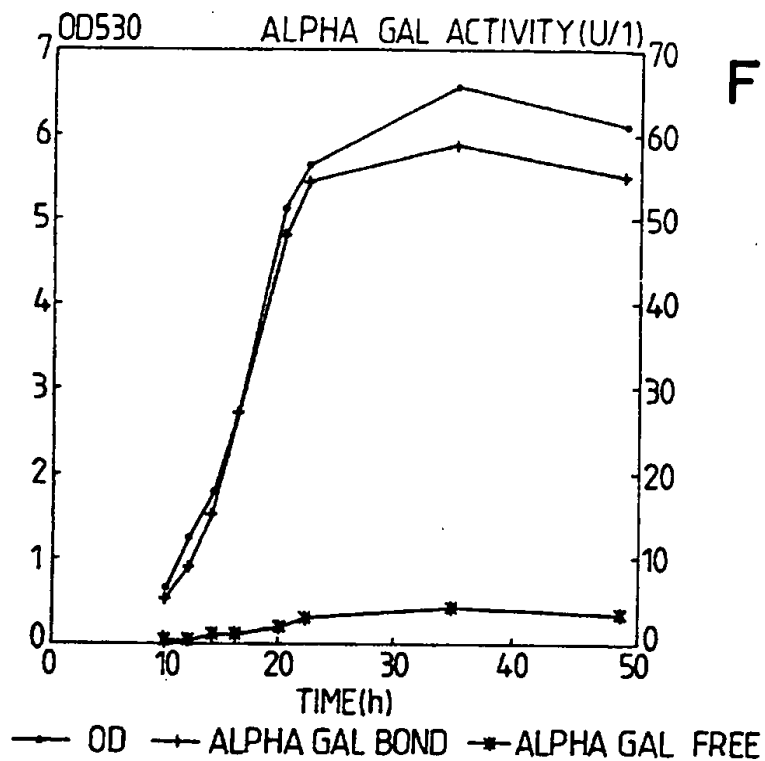
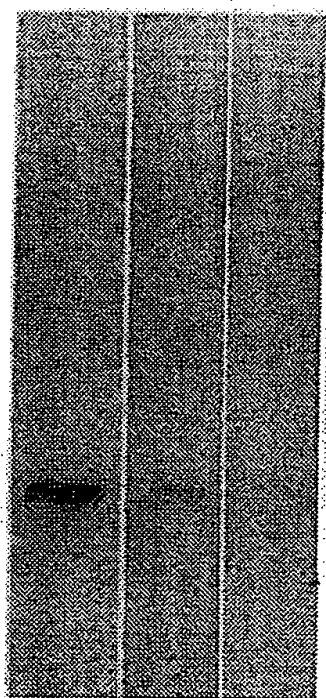
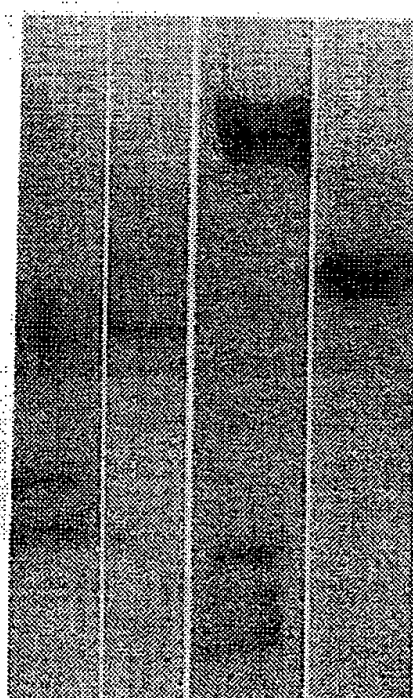


FIG. 5A



1 2 3

FIG. 5B



1 2 3 4

MW
(kD)

— 224

— 109

— 46

— 29

FIG. 6A

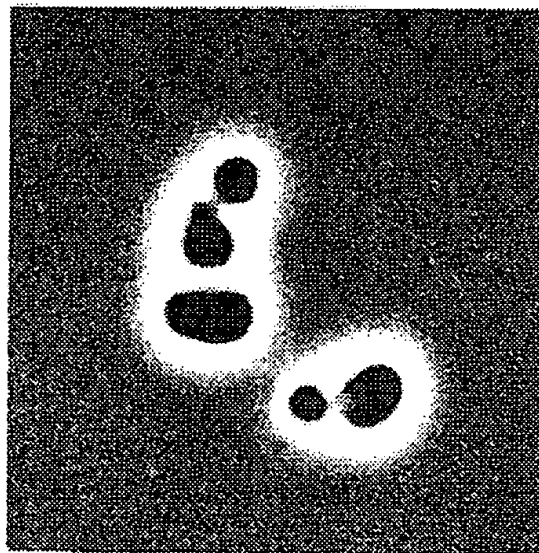
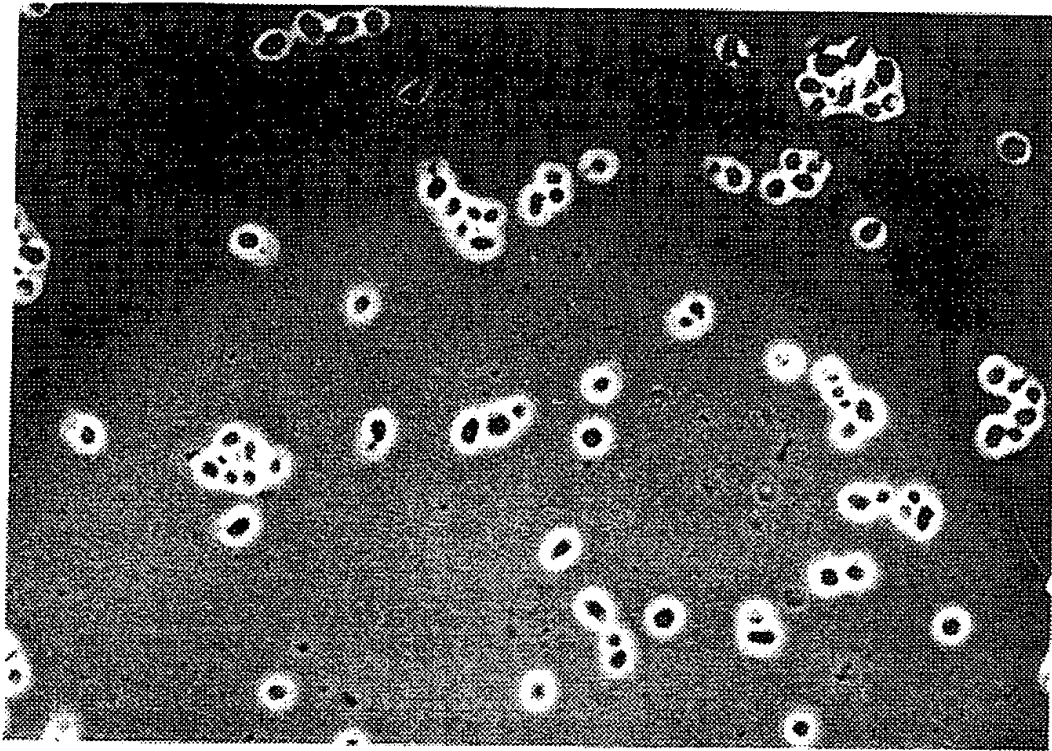
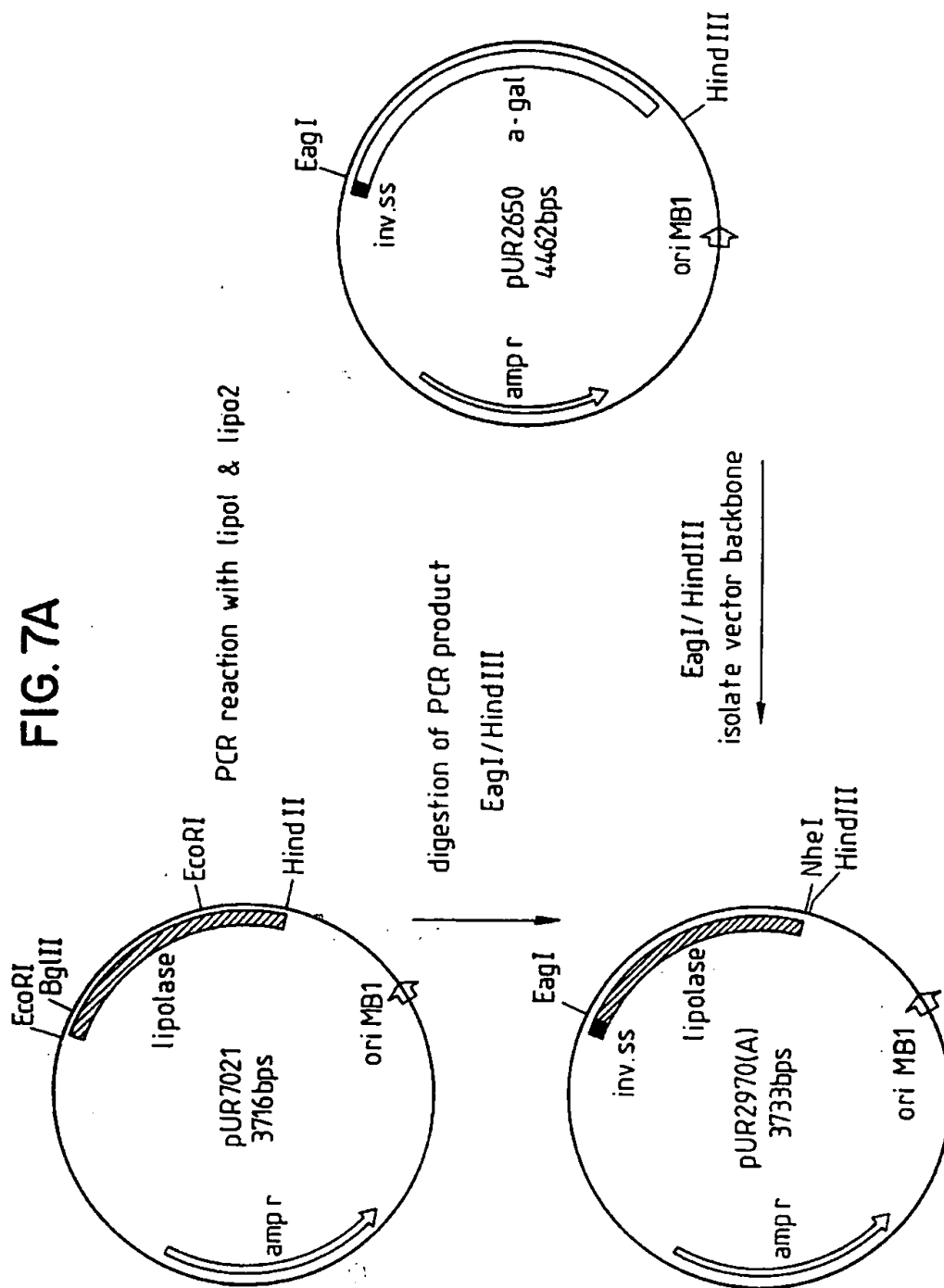


FIG. 6B

FIG. 7A



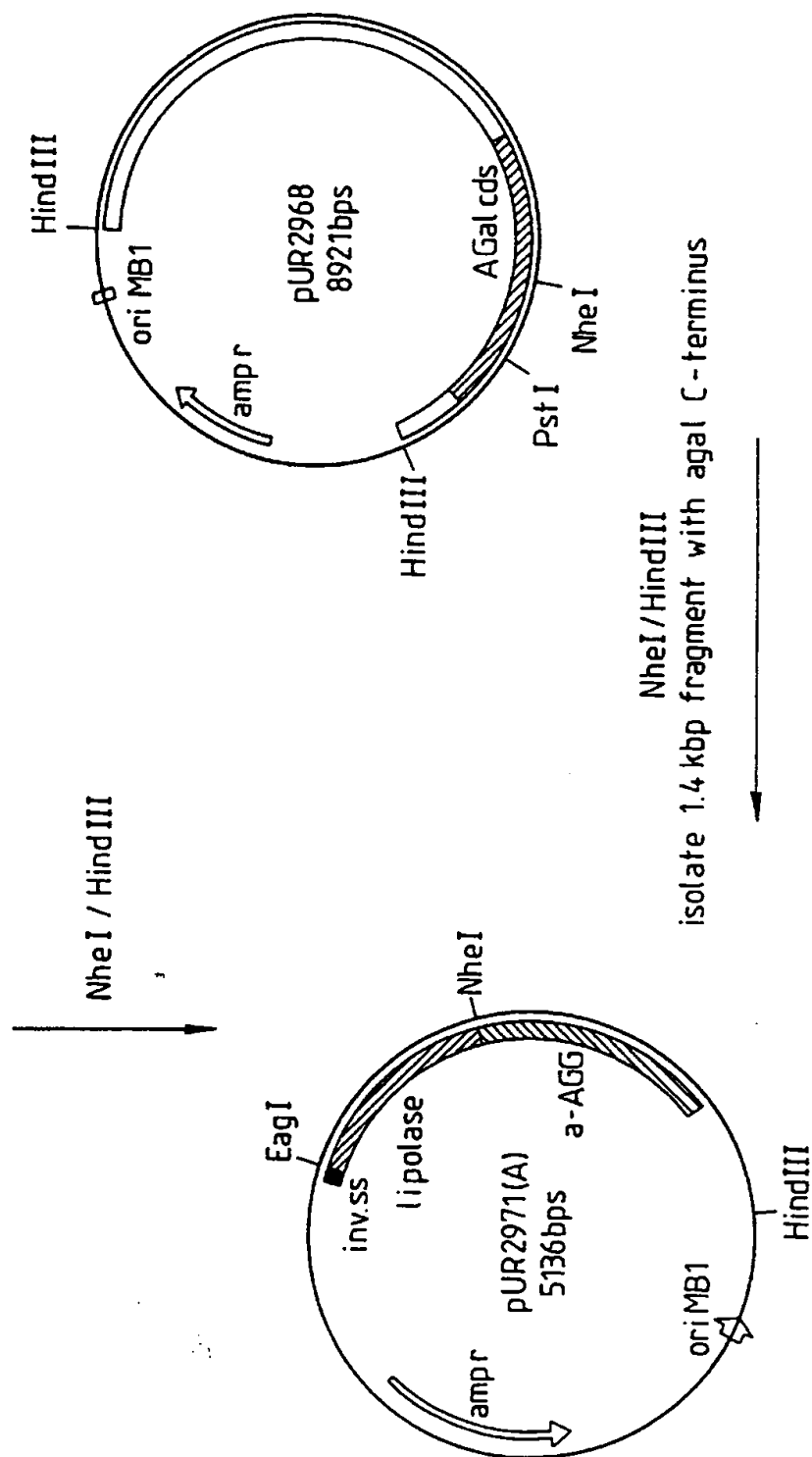
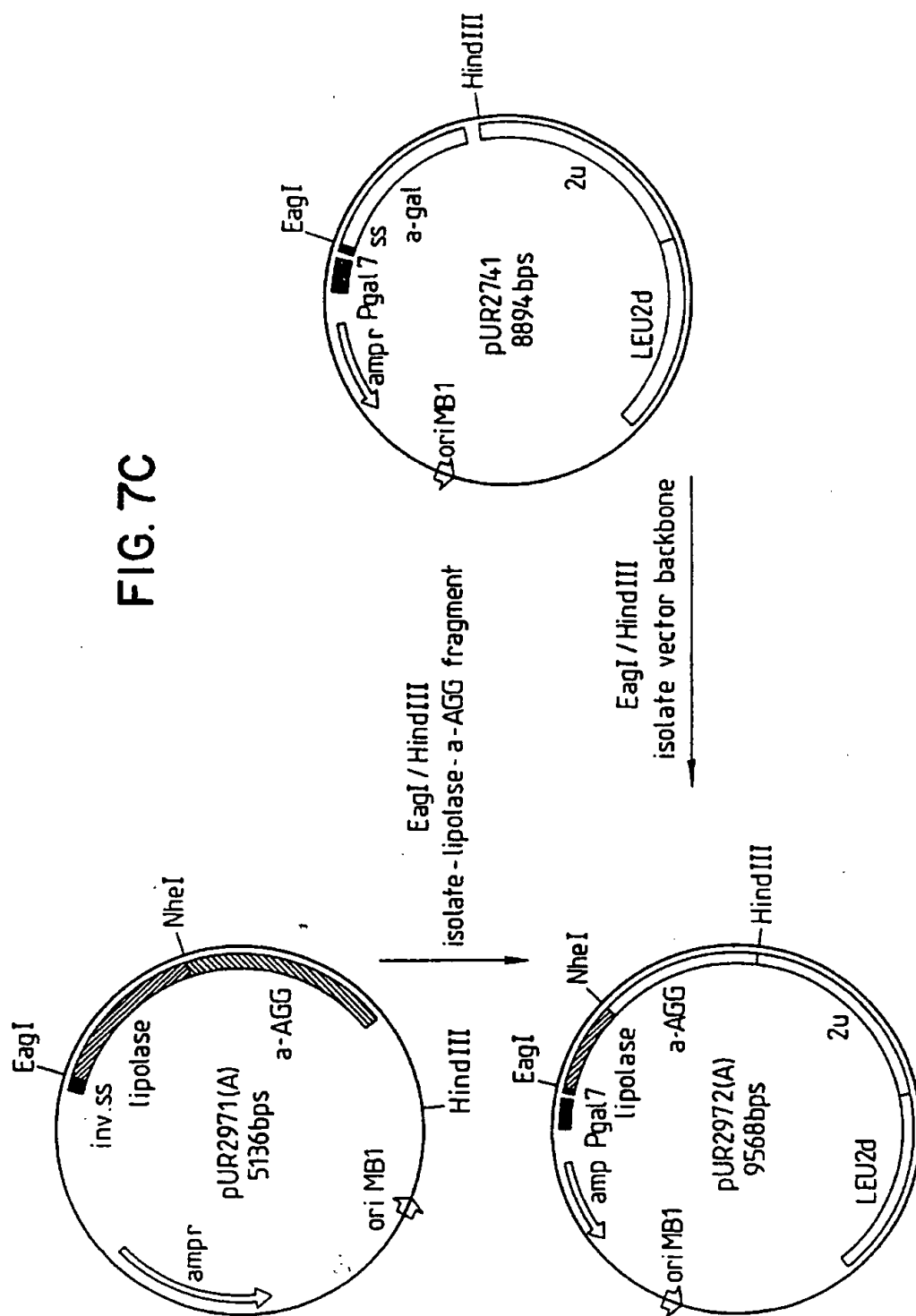


FIG. 7B

FIG. 7C



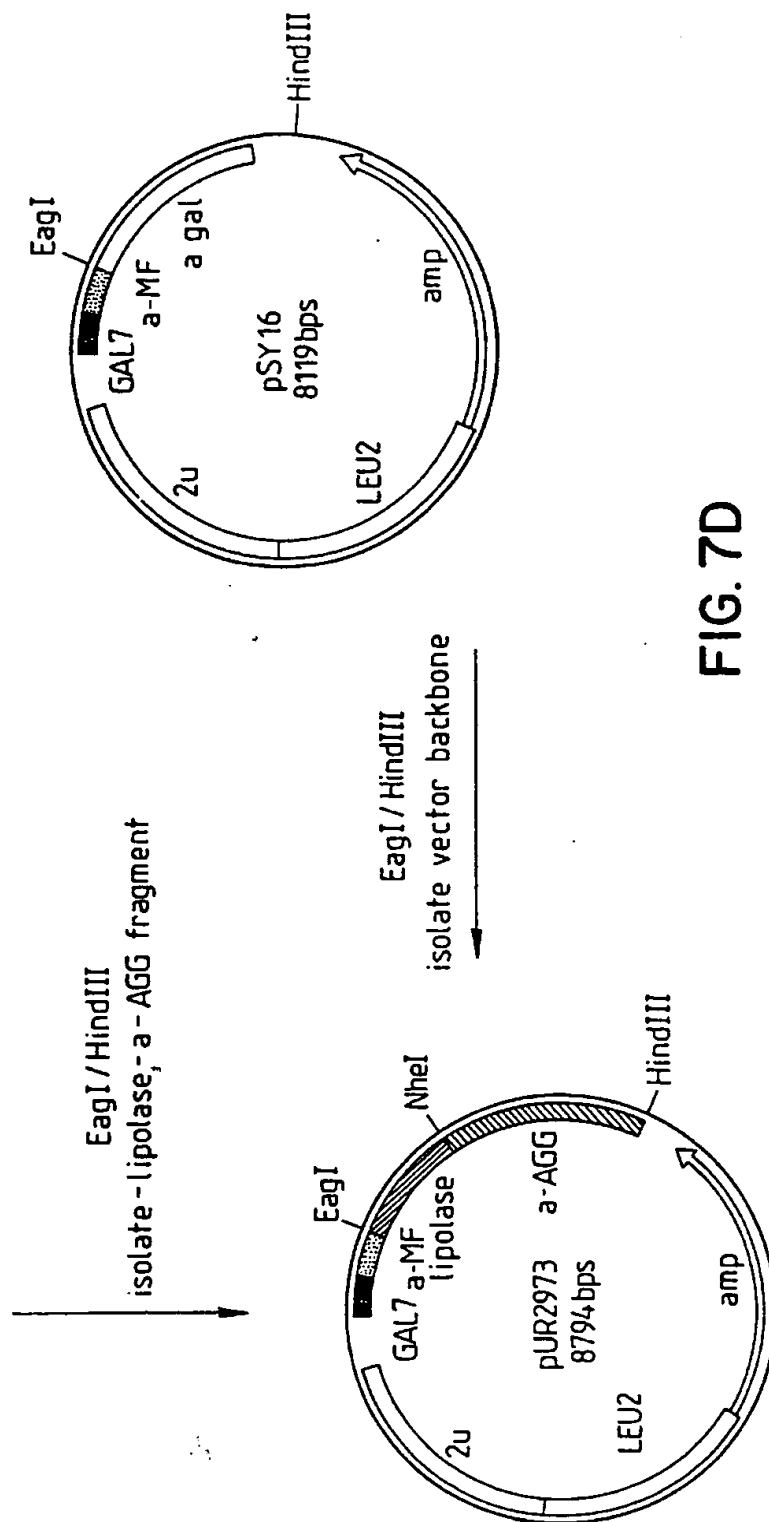


FIG. 7D

FIG. 8A

1	AATTCGGCAC	GAGATTCCTT	TGATTTGCAA	CTGTTAATCA
41	TGGTTTCCAA	AAGCTTTTTT	TTGGCTGCGG	CGCTCAACGT
81	AGTGGGCACC	TTGGCCCAGG	CCCCCACGGC	CGTTCCTAAT
121	GGCAACGAGG	TCATCTCTGG	TGTCCTTGAG	GGCAAGGTTG
161	ATACCTTCAA	GGGAATCCCA	TTTGCTGACC	CTCCTGTTGG
201	TGACTTGCGG	TTCAAGCACC	CCCAGCCTTT	CACTGGATCC
241	TACCAGGGTC	TTAAGGCCAA	CGACTTCAGC	TCTGCTTGTA
281	TGCAGCTTGA	TCCTGGCAAT	GCCTTTTCTT	TGCTTGACAA
321	AGTAGTGGGC	TTGGGAAAGA	TTCTTCCTGA	TAACCTTAGA
361	GGCCCTCTTT	ATGACATGGC	CCAGGGTAGT	GTCTCCATGA
401	ATGAGGACTG	TCTCTACCTT	AACGTTTTCC	GCCCCGCTGG
441	CACCAAGCCT	GATGCTAAGC	TCCCCGTCAT	GGTTTGGATT
481	TACGGTGGTG	CCTTTGTGTT	TGGTTCTTCT	GCTTCTTACC
521	CTGGTAACGG	CTACGTCAAG	GAGAGTGTGG	AAATGGGCCA
561	GCCTGTTGTG	TTTGTTTCCA	TCAACTACCG	TACCGGCCCC
601	TATGGATTCT	TGGGTGGTGA	TGCCATCACC	GCTGAGGGCA
641	ACACCAACGC	TGGTCTGCAC	GACCAGCGCA	AGGGTCTCGA
681	GTGGGTTAGC	GACAACATTG	CCAACTTTGG	TGGTGATCCC
721	GACAAGGTCA	TGATTTTCGG	TGAGTCCGCT	GGTGCCATGA
761	GTGTTGCTCA	CCAGCTTGTT	GCCTACGGTG	GTGACAACAC
801	CTACAACGGA	AAGCAGCTTT	TCCACTCTGC	CATTCTTCAG
841	TCTGGCGGTC	CTCTTCCTTA	CTTTGACTCT	ACTTCTGTTG
881	GTCCCGAGAG	TGCCTACAGC	AGATTTGCTC	AGTATGCCGG
921	ATGTGACACC	AGTGCCAGTG	ATAATGACAC	TCTGGCTTGT
961	CTCCGCAGCA	AGTCCAGCGA	TGTCTTGCAC	AGTGCGCAGA
1001	ACTCGTATGA	TCTTAAGGAC	CTGTTTGGTC	TGCTCCCTCA
1041	ATTCCTTGGA	TTTGGTCCCA	GACCCGACGG	CAACATTATT
1081	CCCGATGCCG	CTTATGAGCT	CTACCGCAGC	GGTAGATACG
1121	CCAAGGTTCC	CTACATTACT	GGCAACCAGG	AGGATGAGGG
1161	TACTATTCTT	GCCCCCGTTG	CTATTAATGC	TACCACTACT
1201	CCCCATGTTA	AGAAGTGGTT	GAAGTACATT	TGTAGCCAGG
1241	CTTCTGACGC	TTCGCTTGAT	CGTGTTTTGT	CGCTCTACCC
1281	CGGCTCTTGG	TCGGAGGGTT	CACCATTCGG	CACTGGTATT
1321	CTTAATGCTC	TTACCCCTCA	GTTCAAGCGC	ATTGCTGCCA
1361	TTTTCACTGA	TTTGCTGTTC	CAGTCTCCTC	GTCGTGTTAT
1401	GCTTAACGCT	ACCAAGGACG	TCAACCGCTG	GACTTACCTT
1441	GCCACCCAGC	TCCATAACCT	CGTTCCATTT	TTGGGTACTT
1481	TCCATGGCAG	TGATCTTCTT	TTTCAATACT	ACGTGGACCT
1521	TGGCCCATCT	TCTGCTTACC	GCCGCTACTT	TATCTCGTTT
1561	GCCAACCACC	ACGACCCCAA	CGTTGGTACC	AACCTCCAAC

FIG. 8B

1601 AGTGGGATAT GTACACTGAT GCAGGCAAGG AGATGCTTCA
1641 GATTCATATG ATTGGTAACT CTATGAGAAC TGACGACTTT
1681 AGAATCGAGG GAATCTCGAA CTTTGAGTCT GACGTTACTC
1721 TCTTCGGTTA ATCCCATTTA GCAAGTTTTG TGTATTTCAA
1761 GTATACCAGT TGATGTAATA TATCAATAGA TTACAAATTA
1801 ATTAGTGAAA AAAAAAAAAA AAAAAAAC 1828

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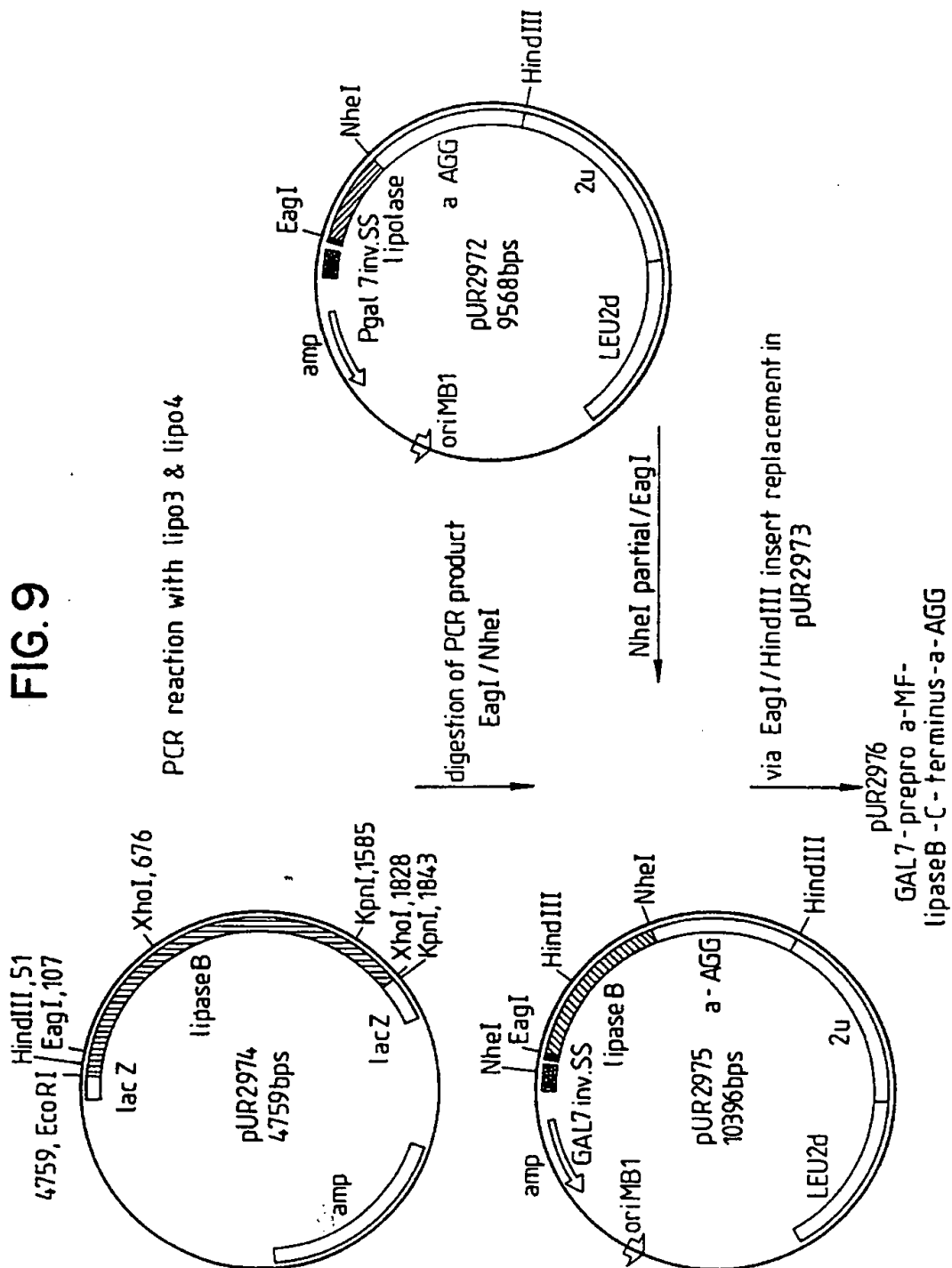
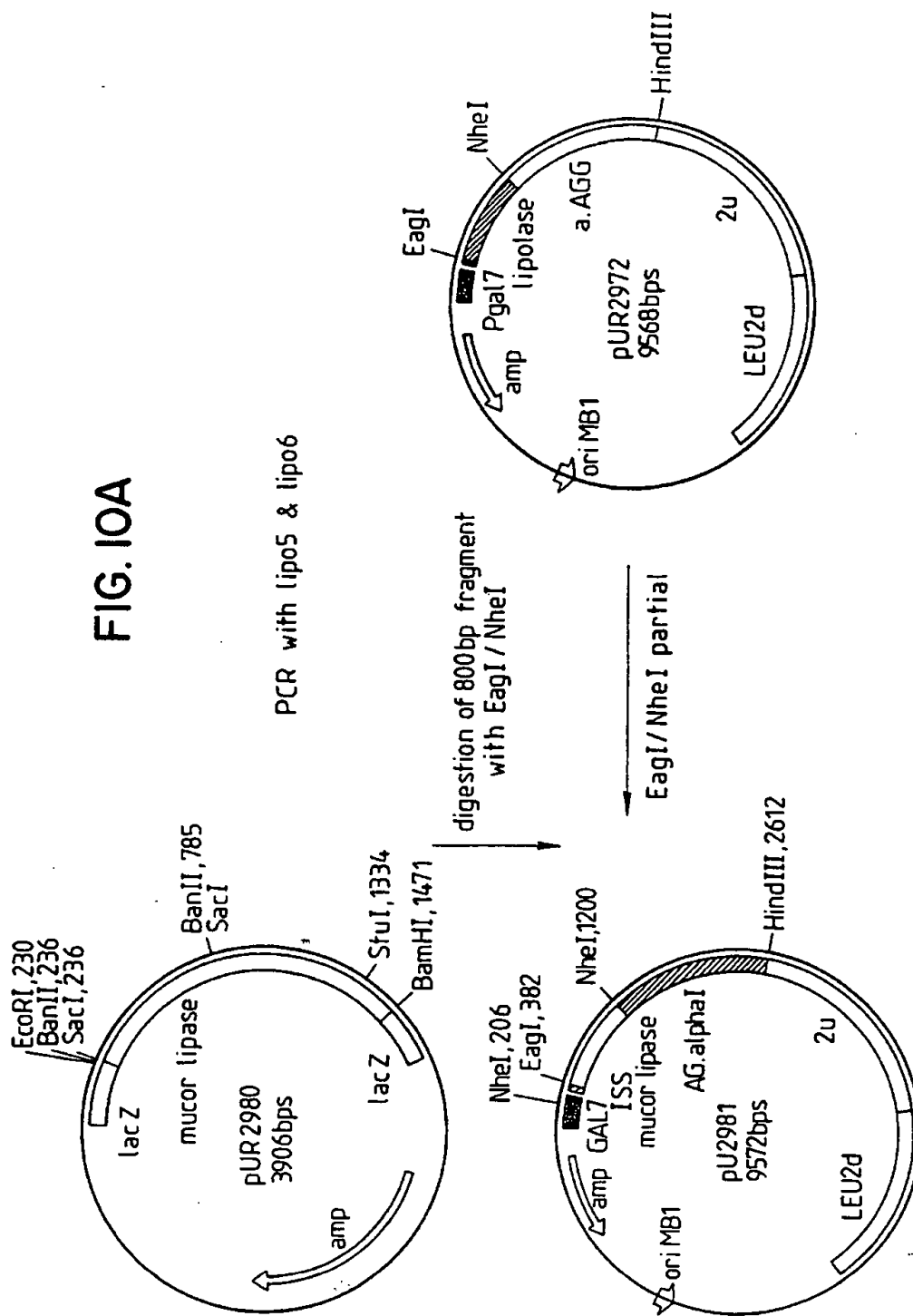


FIG. 10A



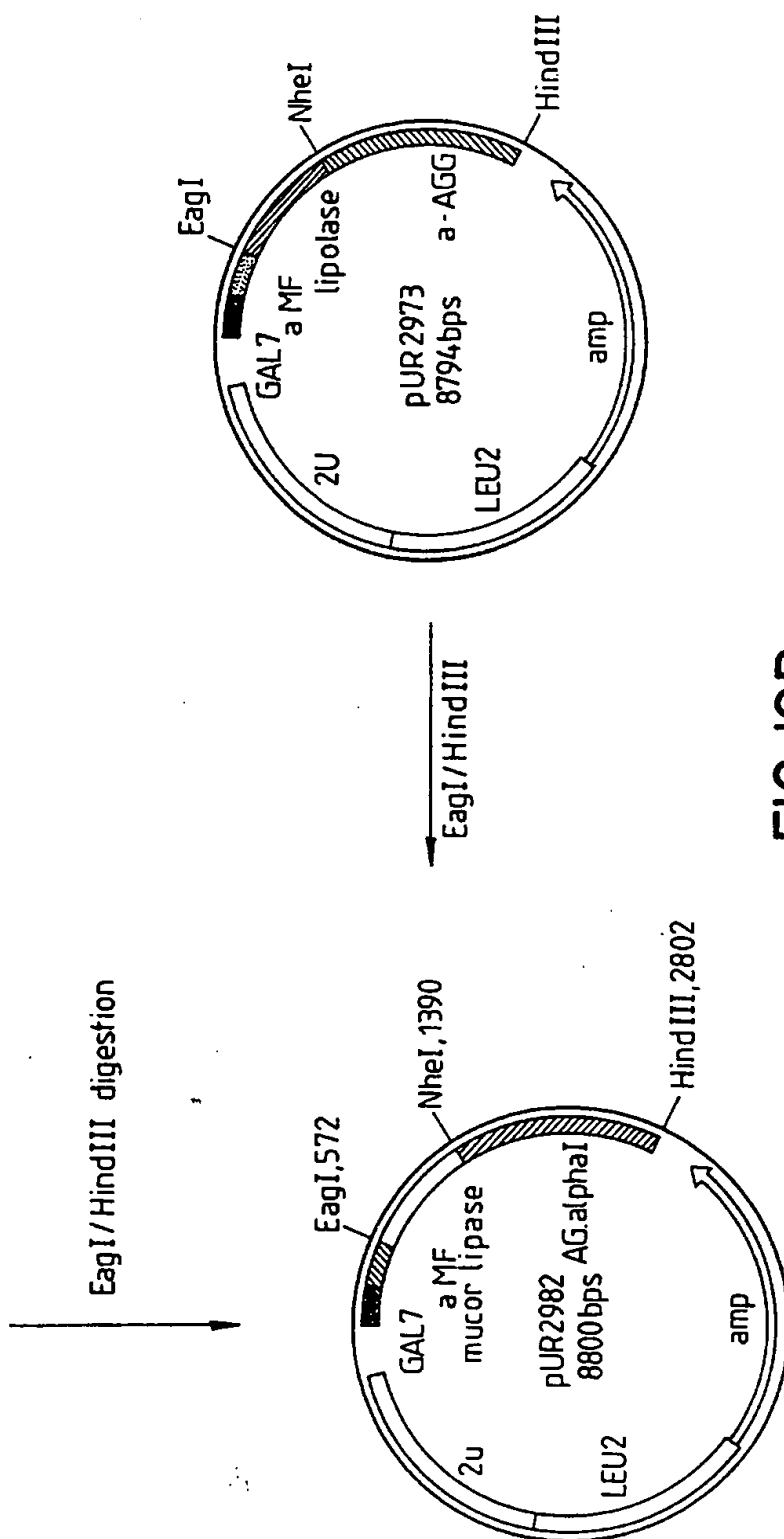


FIG. 10B

FIG. IIA

1	ATGACAATGC	CTCATCGCTA	TATGTTTTTG	GCAGTCTTTA
41	CACTTCTGGC	ACTAACTAGT	GTGGCCTCAG	GAGCCACAGA
81	GGCGTGCTTA	CCAGCAGGCC	AGAGGAAAAG	TGGGATGAAT
121	ATAAATTTTT	ACCAGTATTC	ATTGAAAGAT	TCCTCCACAT
161	ATTCGAATGC	AGCATATATG	GCTTATGGAT	ATGCCTCAAA
201	AACCAAATA	GGTTCTGTCT	GAGGACAAAC	TGATATCTCG
241	ATTGATTATA	ATATTCCCTG	TGTTAGTTCA	TCAGGCACAT
281	TTCCTTGTC	TCAAGAAGAT	TCCTATGGAA	ACTGGGGATG
321	CAAAGGAATG	GGTGCTTGTT	CTAATAGTCA	AGGAATTGCA
361	TACTGGAGTA	CTGATTTATT	TGGTTTCTAT	ACTACCCCAA
401	CAAACGTAA	CCTAGAAATG	ACAGGTATT	TTTTACCACC
441	ACAGACGGGT	TCTTACACAT	TCAAGTTTGC	TACAGTTGAC
481	GACTCTGCAA	TTCTATCAGT	AGGTGGTGCA	ACCGCGTTCA
521	ACTGTTGTGC	TCAACAGCAA	CCGCCGATCA	CATCAACGAA
561	CTTTACCATT	GACGGTATCA	AGCCATGGGG	TGGAAGTTTG
601	CCACCTAATA	TCGAAGGAAC	CGTCTATATG	TACGCTGGCT
641	ACTATTATCC	AATGAAGGTT	GTTTACTCGA	ACGCTGTTTC
681	TTGGGGTACA	CTTCCAATTA	GTGTGACACT	TCCAGATGGT
721	ACCACTGTAA	GTGATGACTT	CGAAGGGTAC	GTCTATTCTT
761	TTGACGATGA	CCTAAGTCAA	TCTAACTGTA	CTGTCCCTGA
801	CCCTTCAAAT	TATGCTGTCA	GTACCACTAC	AACTACAACG
841	GAACCATGGA	CCGGTACTTT	CACTTCTACA	TCTACTGAAA
881	TGACCACCGT	CACCGGTACC	AACGGCGTTC	CAACTGACGA
921	AACCGTCATT	GTCATCAGAA	CTCCAACCAG	TGAAGGTCTA
961	ATCAGCACCA	CCACTGAACC	ATGGACTGGC	ACTTTCACTT
1001	CGACTTCCAC	TGAGGTTACC	ACCATCACTG	GAACCAACGG
1041	TCAACCAACT	GACGAAACTG	TGATTGTTAT	CAGAACTCCA
1081	ACCAGTGAAG	GTCTAATCAG	CACCACCACT	GAACCATGGA
1121	CTGGTACTTT	CACTTCTACA	TCTACTGAAA	TGACCACCGT
1161	CACCGGTACT	AACGGTCAAC	CAACTGACGA	AACCGTGATT
1201	GTTATCAGAA	CTCCAACCAG	TGAAGGTTTG	GTTACAACCA
1241	CCACTGAACC	ATGGACTGGT	ACTTTTACTT	CGACTTCCAC
1281	TGAAATGTCT	ACTGTCAGTG	GAACCAATGG	CTTGCCAACT
1321	GATGAAACTG	TCATTGTTGT	CAAACTCCA	ACTACTGCCA
1361	TCTCATCCAG	TTTGTCATCA	TCATCTTCAG	GACAAATCAC
1401	CAGCTCTATC	ACGTCTTCGC	GTCCAATTAT	TACCCCATTC
1441	TATCCTAGCA	ATGGAACCTC	TGTGATTTCT	TCCTCAGTAA
1481	TTTCTTCCTC	AGTCACTTCT	TCTCTATTCA	CTTCTTCTCC
1521	AGTCATTTCT	TCCTCAGTCA	TTTCTTCTTC	TACAACAACC
1561	TCCAATTCTA	TATTTTCTGA	ATCATCTAAA	TCATCCGTCA

FIG. IIB

1601	TTCCAACCAG	TAGTTCCACC	TCTGGTTCTT	CTGAGAGCGA
1641	AACGAGTTCA	GCTGGTTCTG	TCTCTTCTTC	CTCTTTTATC
1681	TCTTCTGAAT	CATCAAAATC	TCCTACATAT	TCTTCTTCAT
1721	CATTACCACT	TGTTACCACT	GCGACAACAA	GCCAGGAAAC
1761	TGCTTCTTCA	TTACCACCTG	CTACCACTAC	AAAAACGAGC
1801	GAACAAACCA	CTTTGGTTAC	CGTGACATCC	TGCGAGTCTC
1841	ATGTGTGCAC	TGAATCCATC	TCCCCTGCGA	TTGTTTCCAC
1881	AGCTACTGTT	ACTGTTAGCG	GCGTCACAAC	AGAGTATACC
1921	ACATGGTGCC	CTATTTCTAC	TACAGAGACA	ACAAAGCAAA
1961	CCAAAGGGAC	AACAGAGCAA	ACCACAGAAA	CAACAAAACA
2001	AACCACGGTA	GTTACAATTT	CTTCTTGTGA	ATCTGACGTA
2041	TGCTCTAAGA	CTGCTTCTCC	AGCCATTGTA	TCTACAAGCA
2081	CTGCTACTAT	TAACGGCGTT	ACTACAGAAT	ACACAACATG
2121	GTGTCCTATT	TCCACCACAG	AATCGAGGCA	ACAAACAACG
2161	CTAGTTACTG	TTACTTCCTG	CGAATCTGGT	GTGTGTTCCG
2201	AAACTGCTTC	ACCTGCCATT	GTTTCGACGG	CCACGGCTAC
2241	TGTGAATGAT	GTTGTTACGG	TCTATCCTAC	ATGGAGGCCA
2281	CAGACTGCGA	ATGAAGAGTC	TGTCAGCTCT	AAAATGAACA
2321	GTGCTACCGG	TGAGACAACA	ACCAATACTT	TAGCTGCTGA
2361	AACGACTACC	AATACTGTAG	CTGCTGAGAC	GATTACCAAT
2401	ACTGGAGCTG	CTGAGACGAA	AACAGTAGTC	ACCTCTTCGC
2441	TTTCAAGATC	TAATCACGCT	GAAACACAGA	CGGCTTCCGC
2481	GACCGATGTG	ATTGGTCACA	GCAGTAGTGT	TGTTTCTGTA
2521	TCCGAAACTG	GCAACACCAA	GAGTCTAACA	AGTTCCGGGT
2561	TGAGTACTAT	GTCGCAACAG	CCTCGTAGCA	CACCAGCAAG
2601	CAGCATGGTA	GGATATAGTA	CAGCTTCTTT	AGAAATTTCA
2641	ACGTATGCTG	GCAGTGCAAC	AGCTTACTGG	CCGGTAGTGG
2681	TTTAA	2685		

FIG.12

PCR with oligonucleotides pcrflo1 & pcrflo2
Isolate 1950 bp fragment
cut with NheI and HindIII
ligate into HindIII/ NheI (p) digested pUR2972

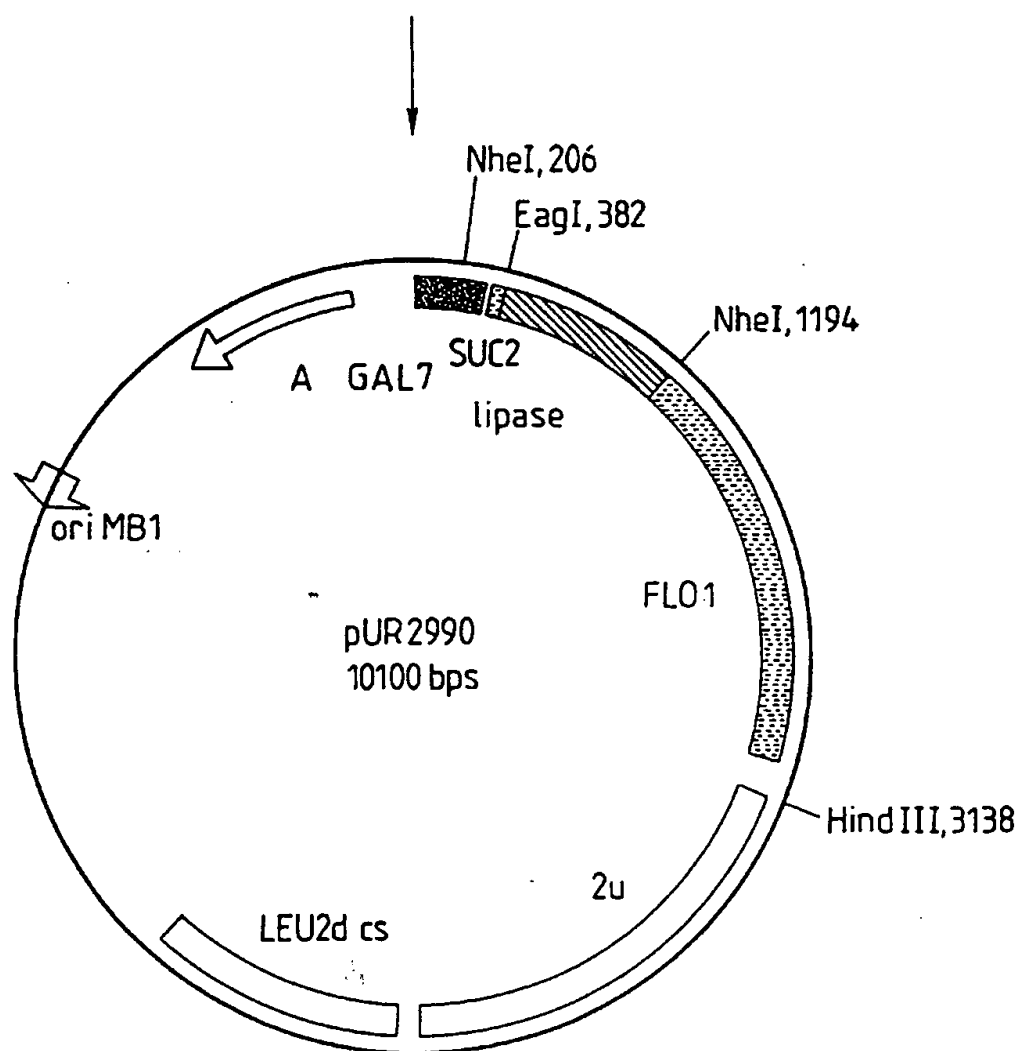


FIG. 13

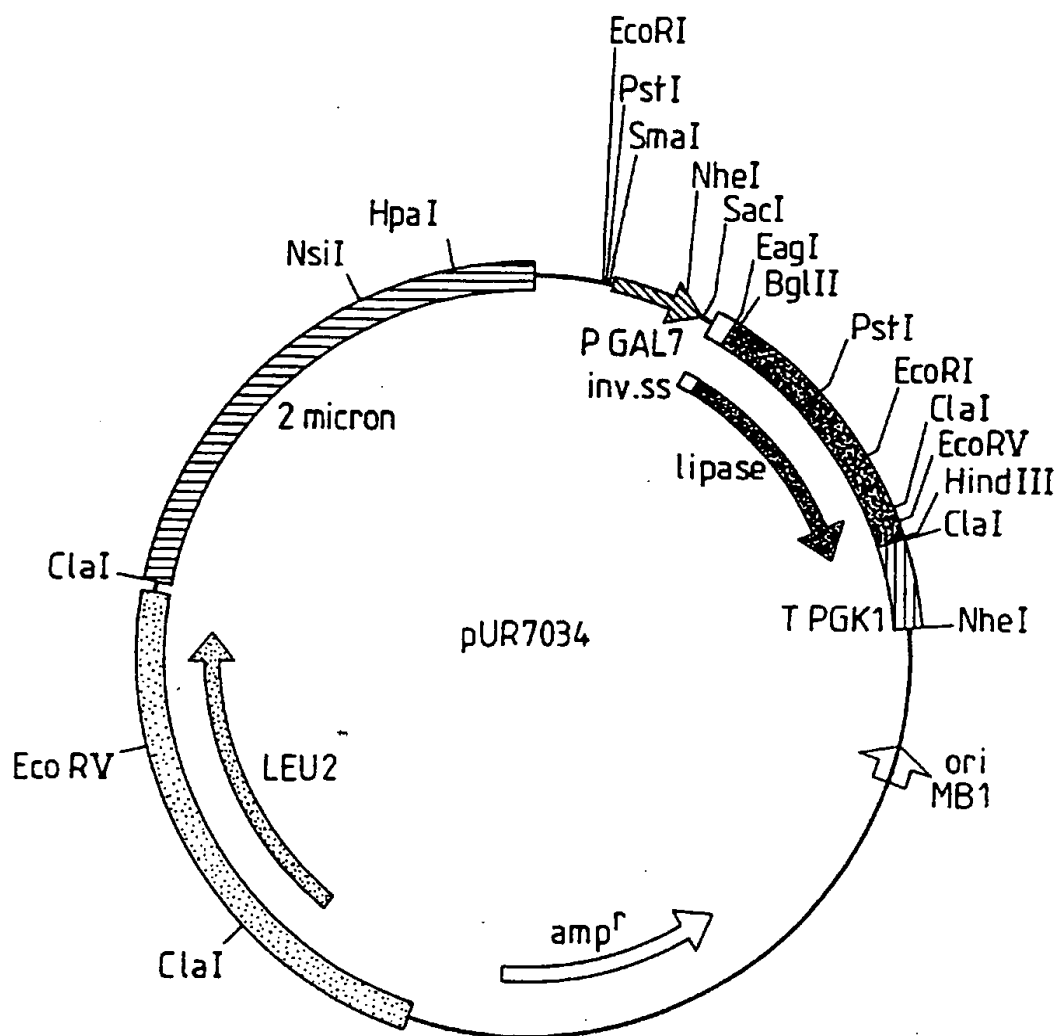


FIG. 14

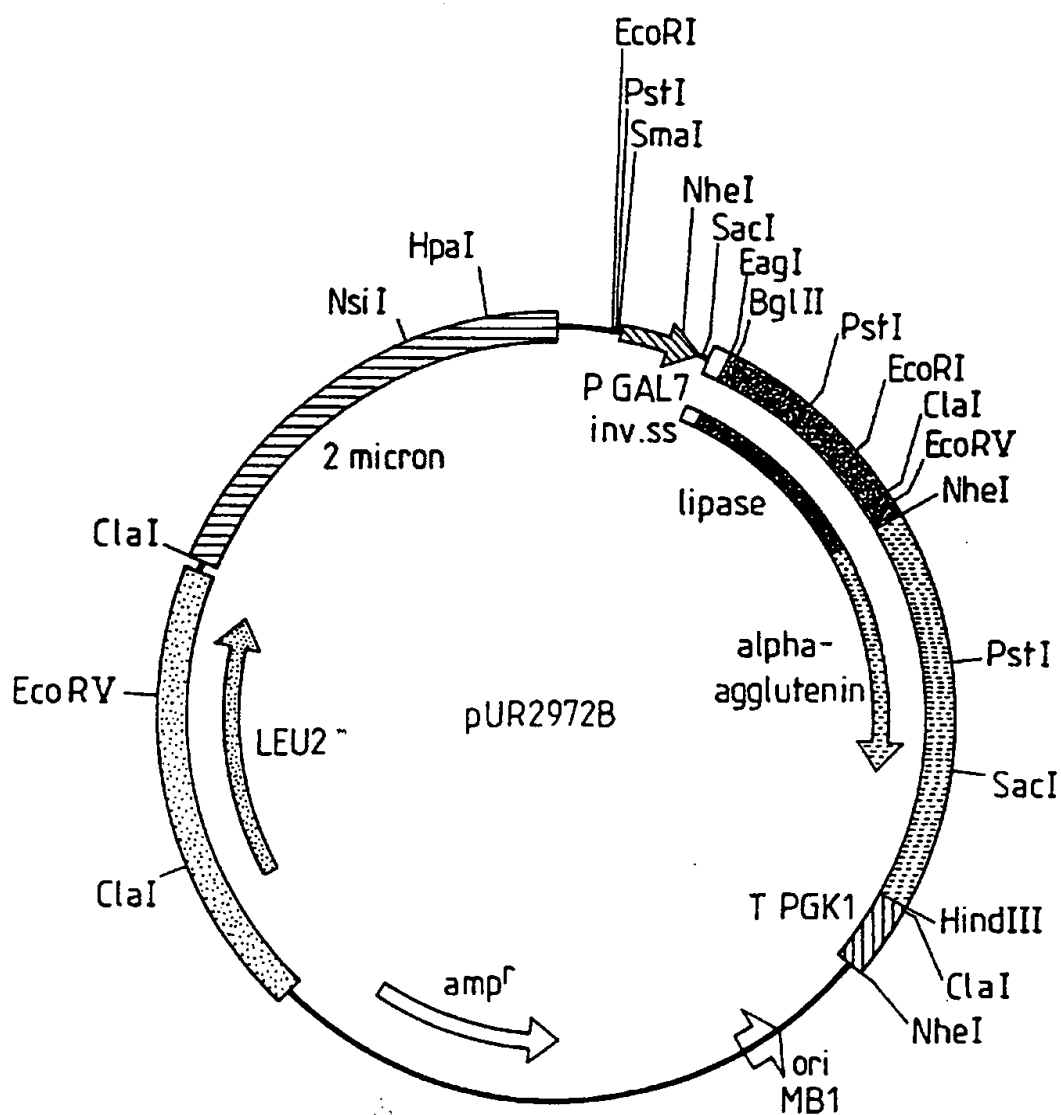


FIG. 15A

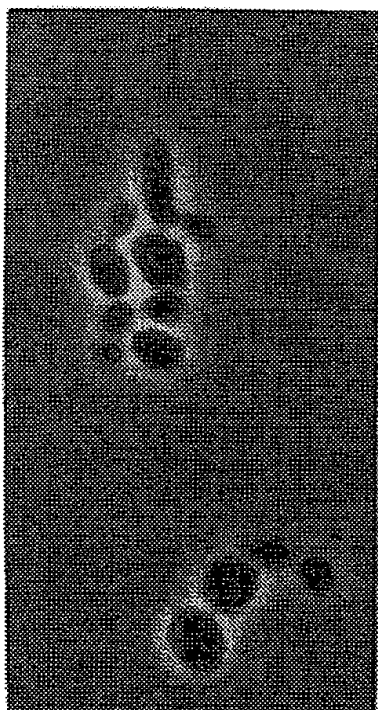
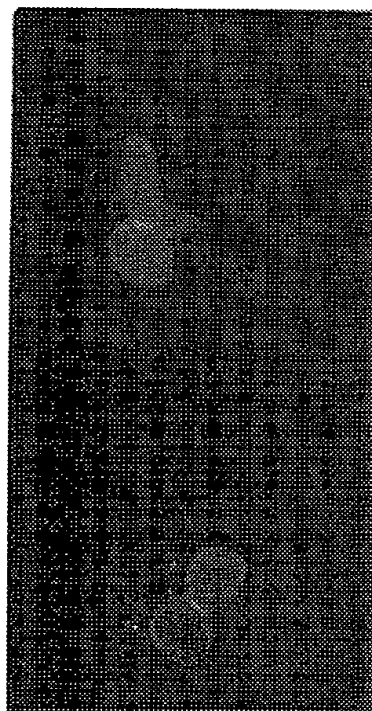


FIG. 15B



PROCESS FOR IMMOBILIZING ENZYMES TO THE CELL WALL OF A MICROBIAL CELL BY PRODUCING A FUSION PROTEIN

The present invention is in the field of conversion processes using immobilized enzymes, produced by genetic engineering.

BACKGROUND OF THE INVENTION

In the detergent, personal care and food products industry there is a strong trend towards natural ingredients of these products and to environmentally acceptable production processes. Enzymic conversions are very important for fulfilling these consumer demands, as these processes can be completely natural. Moreover enzymic processes are very specific and consequently will produce minimum amounts of waste products. Such processes can be carried out in water at mild temperatures and atmospheric pressure. However enzymic processes based on free enzymes are either quite expensive due to the loss of enzymes or require expensive equipment, like ultra-membrane systems to entrap the enzyme.

Alternatively enzymes can be immobilized either physically or chemically. The latter method has often the disadvantage that coupling is carried out using non-natural chemicals and in processes that are not attractive from an environmental point of view. Moreover chemical modification of enzymes is nearly always not very specific, which means that coupling can affect the activity of the enzyme negatively. Physical immobilization can comply with consumer demands, however also physical immobilization may affect the activity of the enzyme in a negative way. Moreover, a physically immobilized enzyme is in equilibrium with free enzyme, which means that in continuous reactors, according to the laws of thermodynamics, substantial losses of enzyme are unavoidable.

There are a few publications on immobilization of enzymes to microbial cells (see reference 1). The present invention provides a method for immobilizing enzymes to cell walls of microbial cells in a very precise way. Additionally, the immobilization does not require any chemical or physical coupling step and is very efficient. Some extracellular proteins are known to have special functions which they can perform only if they remain bound to the cell wall of the host cell. Often this type of protein has a long C-terminal part that anchors it in the cell wall. These C-terminal parts have very special amino acid sequences. A typical example is anchoring via C-terminal sequences enriched in proline (see reference 2). Another mechanism to anchor proteins in cell walls is that the protein has a glycosyl-phosphatidyl-inositol (GPI) anchor (see reference 3) and that the C-terminal part of the protein contains a substantial number of potential serine and threonine glycosylation sites. O-Glycosylation of these sites gives a rod-like conformation to the C-terminal part of these proteins. Another feature of these manno-proteins is that they seem to be linked to the glucan in the cell wall of lower eukaryotes, as they cannot be extracted from the cell wall with SDS, but can be liberated by glucanase treatment.

SUMMARY OF THE INVENTION

The present invention provides a method for immobilizing an enzyme, which comprises the use of recombinant DNA techniques for producing an enzyme or a functional part thereof linked to the cell wall of a host cell, preferably a microbial cell, and whereby the enzyme or functional

fragment thereof is localized at the exterior of the cell wall. Preferably the enzyme or the functional part thereof is immobilized by linking to the C-terminal part of a protein that ensures anchoring in the cell wall.

In one embodiment of the invention a recombinant polynucleotide is provided comprising a structural gene encoding a protein providing catalytic activity and at least a part of a gene encoding a protein capable of anchoring in a eukaryotic or prokaryotic cell wall, said part encoding at least the C-terminal part of said anchoring protein. Preferably the polynucleotide further comprises a sequence encoding a signal peptide ensuring secretion of the expression product of the polynucleotide. Such signal peptide can be derived from a glycosyl-phosphatidyl-inositol (GPI) anchoring protein, α -factor, α -agglutinin, invertase or inulinase, α -amylase of *Bacillus*, or a proteinase of lactic acid bacteria. The DNA sequence encoding a protein capable of anchoring in the cell wall can encode α -agglutinin, AGA1 (α -agglutinin) FLO1 (flocculation protein) or the Major Cell Wall Protein of lower eukaryotes, or a proteinase of lactic acid bacteria. The recombinant polynucleotide is operably linked to a promoter, preferably an inducible promoter. The DNA sequence encoding a protein providing catalytic activity can encode a hydrolytic enzyme, e.g. a lipase, or an oxidoreductase, e.g. an oxidase. Another embodiment of the invention relates to a recombinant vector comprising a polynucleotide as described above. If such vector contains a DNA sequence encoding a protein providing catalytic activity, which protein exhibits said catalytic activity when present in a multimeric form, said vector can further comprise a second polynucleotide comprising a structural gene encoding the same protein providing catalytic activity combined with a sequence encoding a signal peptide ensuring secretion of the expression product of said second polynucleotide, said second polynucleotide being operably linked to a regulatable promoter, preferably an inducible or repressible promoter.

A further embodiment of the invention relates to a chimeric protein encoded by a polynucleotide as described above.

Still another embodiment is a host cell, preferably a microorganism, containing a polynucleotide as described above or a vector as described above. If the protein providing catalytic activity exhibits said catalytic activity when present in a multimeric form, said host cell or microorganism can further comprise a second polynucleotide comprising a structural gene encoding the same protein providing catalytic activity combined with a sequence encoding a signal peptide ensuring secretion of the expression product of said second polynucleotide, said second polynucleotide being operably linked to a regulatable promoter, preferably an inducible or repressible promoter, and said second polynucleotide being present either in another vector or in the chromosome of said microorganism. Preferably the host cell or microorganism has at least one of said polynucleotides integrated in its chromosome. As a result of culturing such host cell or microorganism the invention provides a host cell, preferably a microorganism, having a protein as described above immobilized on its cell wall. The host cell or microorganism can be a lower eukaryote, in particular a yeast.

The invention also provides a process for carrying out an enzymatic process by using an immobilized catalytically active protein, wherein a substrate for said catalytically active protein is contacted with a host cell or microorganism according to the invention.

BRIEF DESCRIPTION OF THE FIGURES

FIG. 1: DNA sequence of the 6057 bp HindIII fragment containing the complete AGA1 gene of *S. cerevisiae* (see

SEQ ID NO: 1). The position of the unique *NheI* site and the *HindIII* site used for the described constructions is specified in the header.

FIG. 2: Schematic presentation of the construction of pUR2969. The restriction sites for endonucleases used are shown. Abbreviations used: AG- α -1: Gene expressing α -agglutinin from *S. cerevisiae*

amp: β -lactamase resistance gene

PGKp: phosphoglyceratekinase promoter

PGKt: terminator of the same gene.

FIG. 3: α -Galactosidase activity of *S. cerevisiae* MT302/1C cells and culture fluid transformed with pSY13 during batch culture:

A: U/l α -galactosidase per time; the OD₅₃₀ is also shown

B: α -galactosidase activity of free and bond enzyme expressed in U/OD₅₃₀.

FIG. 4: α -Galactosidase activity of *S. cerevisiae* MT302/1C cells and culture fluid transformed with pUR2969 during batch culture:

A: U/l α -galactosidase per time; the OD₅₃₀ is also shown

B: α -galactosidase activity of free and bond enzyme expressed in U/OD₅₃₀.

FIG. 5: Western analysis with anti α -galactosidase serum of extracellular fractions of cells of exponential phase (OD₅₃₀=2). The analyzed fractions are equivalent to 4 mg cell walls, (fresh weight):

A: MT302/1C expressing α -galactosidase,

lane 1, growth medium

lane 2, SDS extract of isolated cell walls

lane 3, glucanase extract of SDS extracted cell walls;

B: MT302/1C expressing α -Gal-AG α 1 fusion protein,

lane 1, growth medium

lane 2, SDS extract of isolated cell walls

lane 3, glucanase extract of SDS-extracted cell walls

lane 4: Endo-H treated glucanase extract.

FIG. 6: Immunofluorescent labelling (anti α -galactosidase) of MT302/1C cells in the exponential phase (OD₅₃₀=2) expressing the α -Gal- α -agglutinin fusion protein.

Phase micrograph of intact cells A: overview B: detail.

FIG. 7: Schematic presentation of the construction of pUR2970A, pUR2971A, pUR2972A, and pUR2973. The restriction sites for endonucleases used are indicated in the figure. PCR oligonucleotide sequences are mentioned in the text.

Abbreviations used: AGal cds: coding sequence of α -agglutinin

a-AGG=AGal: Gene expressing α -agglutinin from *S. cerevisiae*

amp: β -lactamase resistance gene

lipolase: lipase gene of *Humicola*

a-MF: prepro- α -mating factor sequence

Pgal7=GAL7: GAL7 promoter

invSS: SUC2 signal sequence

a-gal: α -galactosidase gene

LEU2d: truncated promoter of LEU2 gene;

LEU2: LEU2 gene with complete promoter sequence.

FIG. 8: DNA sequence of a fragment containing the complete coding sequence of lipase B of *Geotrichum candidum* strain 335426 (see SEQ ID NO: 11). The sequence of the mature lipase B starts at nucleotide 97 of the given sequence. The coding sequence starts at nucleotide 40 (ATG).

FIG. 9: Schematic presentation of the construction of pUR2975 and pUR2976. The restriction sites for endonucleases used are shown. Abbreviations used:

a-AGG: Gene expressing α -agglutinin from *S. cerevisiae*

amp: β -lactamase resistance gene

invSS: SUC2 signal sequence

LEU2d: truncated promoter LEU2 gene

Pgal7=GAL7: GAL7 promoter

a-MF: prepro- α -mating factor sequence

lipolase: lipase gene of *Humicola*

lipaseB: lipaseB gene of *Geotrichum candidum*.

FIG. 10: Schematic presentation of the construction of pUR2981 and pUR2982. The restriction sites for endonucleases used are shown. Abbreviations used:

a-AGG=AG- α 1: Gene expressing α -agglutinin from *S. cerevisiae*

mucor lipase: lipase gene of *Rhizomucor miehei*

Pgal7=GAL7: GAL7 promoter

a-MF: prepro- α -mating factor sequence

amp: β -lactamase resistance gene;

2u: 2 μ m sequence

invSS: SUC2 signal sequence

lipolase: lipase gene of *Humicola*

LEU2d: truncated promoter LEU2 gene

LEU2: LEU2 gene with complete promoter sequence.

FIG. 11: DNA sequence (2685 bases) of the 894 amino acids coding part of the FLO1 gene (see SEQ ID NO: 21), the given sequence starts with the codon for the first amino acid and ends with the stop codon.

FIG. 12: Schematic presentation of plasmid pUR2990. Some restriction sites for endonucleases relevant for the given cloning procedure are shown.

FIG. 13: Schematic presentation of plasmid pUR7034.

FIG. 14: Schematic presentation of plasmid pUR2972B.

FIG. 15: Immunofluorescent labelling (anti-lipolase) of SU10 cells in the exponential phase (OD₅₃₀=0.5) expressing the lipolase/ α -agglutinin fusion protein.

A: phase micrograph B: matching fluorescent micrograph

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides a method for immobilizing an enzyme, comprising immobilizing the enzyme or a functional part thereof to the cell wall of a host cell, preferably a microbial cell, using recombinant DNA techniques. In particular, the C-terminal part of a protein that ensures anchoring in the cell wall is linked to an enzyme or the functional part of an enzyme, in such a way that the enzyme is localized on or just above the cell surface. In this way immobilized enzymes are obtained on the surface of cells. The linkage is performed at gene level and is characterized in that the structural gene coding for the enzyme is coupled to at least part of a gene encoding an anchor-protein in such a way that in the expression product the enzyme is coupled at its C-terminal end to the C-terminal part of an anchor-protein. The chimeric enzyme is preferably preceded by a signal sequence that ensures efficient secretion of the chimeric protein.

Thus the invention relates to a recombinant polynucleotide comprising a structural gene encoding a protein providing catalytic activity and at least a part of a gene encoding a protein capable of anchoring in a eukaryotic or prokaryotic cell wall, said part encoding at least the C-terminal part of

said anchoring protein. The length of the C-terminal part of the anchoring protein may vary. Although the entire structural protein could be used, it is preferred that only a part is used, leading to a more efficient exposure of the enzyme protein in the medium surrounding the cell. The anchoring part of the anchoring protein should preferably be entirely present. As an example, about the C-terminal half of the anchoring protein could be used. Preferably, the polynucleotide further comprises a sequence encoding a signal peptide ensuring secretion of the expression product of the polynucleotide. The signal peptide can be derived from a GPI anchoring protein, α -factor, α -agglutinin, invertase or inulinase, α -amylase of *Bacillus*, or a proteinase of lactic acid bacteria. The protein capable of anchoring in the cell wall is preferably selected from the group of α -agglutinin, AGA1, FLO1 (flocculation protein) or the Major Cell Wall Protein of lower eukaryotes, or a proteinase of lactic acid bacteria. The polynucleotide of the invention is preferably operably linked to a promoter, preferably a regulatable promoter, especially an inducible promoter.

The invention also relates to a recombinant vector containing the polynucleotide as described above, and to a host cell containing this polynucleotide, or this vector. In a particular case, wherein the protein providing catalytic activity exhibits said catalytic activity when present in a multimeric form, such as may be the case with oxidoreductases, dimerisation or multimerisation of the monomers might be a prerequisite for activity. The vector and/or the host cell can then further comprise a second polynucleotide comprising a structural gene encoding the same protein providing catalytic activity combined with a sequence encoding a signal peptide ensuring secretion of the expression product of said second polynucleotide, said second polynucleotide being operably linked to a regulatable promoter, preferably an inducible or repressible promoter. Expression and secretion of the second polynucleotide after expression and secretion of the first polynucleotide will then result in the formation of an active multimer on the exterior of the cell wall. The host cell or microorganism preferably contains the polynucleotide described above, or at least one of said polynucleotides in the case of a combination, integrated in its chromosome.

The present invention relates in particular to lower eukaryotes like yeasts that have very stable cell walls and have proteins that are known to be anchored in the cell wall, e.g. α -agglutinin or the product of gene FLO1. Suitable yeasts belong to the genera *Candida*, *Debaryomyces*, *Hansenula*, *Kluyveromyces*, *Pichia* and *Saccharomyces*. Also fungi, especially *Aspergillus*, *Penicillium* and *Rhizopus* can be used. For certain applications also prokaryotes are applicable.

For yeasts the present invention deals in particular with genes encoding chimeric enzymes consisting of:

- the signal sequence e.g. derived from the α -factor-, the invertase-, the α -agglutinin- or the inulinase genes;
- structural genes encoding hydrolytic enzymes such as α -galactosidase, proteases, peptidases, pectinases, pectylesterase, rhamnogalacturonase, esterases and lipases, or non-hydrolytic enzymes such as oxidases; and
- the C-terminus of typically cell wall bound proteins such as α -agglutinin (see reference 4), AGA1 (see reference 5) and FLO1 (see the non-prior published reference 6).

The expression of these genes can be under the control of a constitutive promoter, but more preferred are regulatable,

i.e. repressible or inducible promoters such as the GAL7 promoter for *Saccharomyces*, or the inulinase promoter for *Kluyveromyces* or the methanol-oxidase promoter for *Hansenula*.

Preferably the constructs are made in such a way that the new genetic information is integrated in a stable way in the chromosome of the host cell.

The invention further relates to a host cell, in particular a microorganism, having the chimeric protein described above immobilized on its cell wall. It further concerns the use of such microorganisms for carrying out an enzymatic process by contacting a substrate for the enzyme with the microorganism. Such a process may be carried out e.g. in a packed column, wherein the microorganisms may be supported on solid particles, or in a stirred reactor. The reaction may be aqueous or non-aqueous. Where necessary, additives necessary for the performance of the enzyme, e.g. a co-factor, may be introduced in the reaction medium.

After repeated usage of the naturally immobilized enzyme system in processes, the performance of the system may decrease. This is caused either by physical denaturation or by chemical poisoning or detachment of the enzyme. A particular feature of the present invention is that after usage the system can be recovered from the reaction medium by simple centrifugation or membrane filtration techniques and that the thus collected cells can be transferred to a recovery medium in which the cells revive quickly and concomitantly produce the chimeric protein, thus ensuring that the surface of the cells will be covered by fully active immobilized enzyme. This regeneration process is simple and cheap and therefore will improve the economics of enzymic processes and may result in a much wider application of processes based on immobilized enzyme systems.

However, by no means the present invention is restricted to the reusability of the immobilized enzymes.

The invention will be illustrated by the following examples without the scope of the invention being limited thereto.

EXAMPLE 1

Immobilized α -Galactosidase/ α -Agglutinin on the Surface of *S. cerevisiae*.

The gene encoding α -agglutinin has been described by Lipke et al. (see reference 4). The sequence of a 6057 bp HindIII insert in pTZ18R, containing the whole AGA1 gene is given in FIG. 1. The coding sequence expands over 650 amino acids, including a putative signal sequence starting at nucleotide 3653 with ATG. The unique NheI site cuts the DNA at position 988 of the given coding sequence within the coding part of amino acid 330, thereby separating the α -agglutinin into an N-terminal and a C-terminal part of about same size.

Through digestion of pUR2968 (see FIG. 2) with NheI/HindIII a 1.4 kb fragment was released, containing the sequence information of the putative cell wall anchor. For the fusion to α -galactosidase the plasmid pSY16 was used, an episomal vector based on YEplac 181, harboring the α -galactosidase sequence preceded by the SUC2 invertase signal sequence and placed between the constitutive PGK promoter and PGK terminator. The StyI site, present in the last nine base-pairs of the open reading frame of the α -galactosidase gene, was ligated to the NheI site of the AGA1 gene fragment. To ensure the in frame fusion, the StyI site was filled in and the 5' overhang of the NheI site was removed, prior to ligation into the StyI/HindIII digested pSY13 (see FIG. 2).

To verify the correct assembly of the new plasmid, the shuttle vector was transformed into *E. coli* JM109 (recA1 supE44 endA1 hsdR17 gyrA96 relA1 thi Δ (lac-proAB) F' [traD36 proAB⁺ lacI^q lacZ Δ M15]) (see reference 7) by the transformation protocol described by Chung et al. (see reference 8). One of the positive clones, designated pUR2969, was further characterized, the DNA isolated and purified according to the Quiagen protocol and subsequently characterized by DNA sequencing. DNA sequencing was mainly performed as described by Sanger et al. (see reference 9), and Hsiao (see reference 10), here with the Sequenase version 2.0 kit from United States Biochemical Company, according to the protocol with T7 DNA polymerase (Amersham International plc) and [³⁵S]dATPaS (Amersham International plc: 370 MBq/ml; 22 TBq/mmol).

This plasmid was then transformed into *S. cerevisiae* strain MT302/1C according to the protocol from Klebe et al. (see reference 11).

Yeast transformants were selected on selective plates, lacking leucine, on with 40 μ l (20 mg/ml DMF). X- α -Gal (5-bromo-4-chloro-3-indolyl- α -D-glucose, Boehringer Mannheim) was spread, to directly test for α -galactosidase activity (see reference 12). To demonstrate the expression, secretion, localization and activity of the chimeric protein the following analyses were performed:

1. Expression and Secretion

S. cerevisiae strain MT302/1C was transformed with either plasmid pSY13 containing the α -galactosidase gene of *Cyamopsis tetragonoloba* or plasmid pUR2969 containing the α -galactosidase/ α -agglutinin fusion construct. During batch culture α -galactosidase activities were determined for washed cells and growth medium. The results are given in FIG. 3 and FIG. 4. The α -galactosidase expressed from yeast cells containing plasmid pSY13 was almost exclusively present in the growth medium (FIG. 3A), whereas the α -galactosidase- α -agglutinin fusion protein was almost exclusively cell associated (FIG. 4A). Moreover, the immobilized, cell wall-associated, α -galactosidase- α -agglutinin fusion enzyme had retained the complete activity over the whole incubation time, while the secreted and released enzyme lost about 90% of the activity after an incubation of 65 hours. This indicates, that the immobilization of the described enzyme into the cell wall of yeast protects the enzyme against inactivation, presumably through proteinases, and thereby increases the stability significantly. Further insight into the localization of the different gene products was obtained by Western analysis. Therefore, cells were harvested by centrifugation and washed in 10 mM Tris.HCl, pH 7.8; 1 mM PMSF at 0° C. and all subsequent steps were performed at the same temperature. Three ml isolation buffer and 10 g of glass beads were added per gram of cells (wet weight). The mixture was shaken in a Griffin shaker at 50% of its maximum speed for 30 minutes. The supernatant was isolated and the glass beads were washed with 1M NaCl and 1 mM PMSF until the washes were clear. The supernatant and the washes were pooled. The cell walls were recovered by centrifugation and were subsequently washed in 1 mM PMSF.

Non-covalently bound proteins or proteins bound through disulphide bridges were released from cell walls by boiling for 5 minutes in 50 mM Tris.HCl, pH 7.8; containing 2% SDS, 100 mM EDTA and 40 mM β -mercaptoethanol. The SDS-extracted cell walls were washed several times in 1 mM PMSF to remove SDS. Ten mg of cell walls (wet weight) were taken up in 20 l 100 mM sodium acetate, pH 5.0, containing 1 mM PMSF. To this, 0.5 mU of the β -1,3-glucanase (Laminarase; Sigma L5144) was used as a

source of β -1,3-glucanase) was added followed by incubation for 2 hours at 37° C. Subsequently another 0.5 mU of β -1,3-glucanase was added, followed by incubation for another 2 hours at 37° C.

Proteins were denatured by boiling for 5 minutes preceding Endo-H treatment. Two mg of protein were incubated in 1 ml 50 mM potassium phosphate, pH 5.5, containing 100 mM β -mercaptoethanol and 0.5 mM PMSF with 40 mU Endo-H (Boehringer) for 48 hours at 37° C. Subsequently 20 mU Endo-H were added followed by 24 hours of incubation at 37° C.

Proteins were separated by SDS-PAGE according to Laemmli (see reference 13) in 2.2–20% gradient gels. The gels were blotted by electrophoretic transfer onto Immobilon polyvinylidene-difluoride membrane (Millipore) as described by Towbin et al. (see reference 14). In case of highly glycosylated proteins a subsequently mild periodate treatment was performed in 50 mM periodic acid, 100 mM sodium acetate, pH 4.5, for several hours at 4° C. All subsequent incubations were carried out at room temperature. The blot was blocked in PBS, containing 0.5% gelatine and 0.5% Tween-20, for one hour followed by incubation for 1 hour in probe buffer (PBS, 0.2% gelatine, 0.1% Tween-20) containing 1:200 diluted serum. The blot was subsequently washed several times in washing buffer (PBS; 0.2% gelatine; 0.5% Tween-20) followed by incubation for 1 hour in probe-buffer containing ¹²⁵I-labelled protein A (Amersham). After several washes in washing buffer, the blot was air-dried, wrapped in Saran (Dow) and exposed to X-omat S film (Kodak) with intensifying screen at -70° C. An Omnimedia 6cx scanner and the Adobe Photoshop programme were used to quantify the amount of labelled protein. The results of the various protein isolation procedures from both transformants are given in FIG. 5. While for the transformants comprising the pSY13 plasmid the overall mass of the enzyme was localized in the medium, with only minor amounts of enzyme more entrapped than bond in the cell wall (FIG. 5A)—which could completely be removed by SDS extraction—the fusion protein was tightly bound to the cell wall; with only small amounts of α -galactosidase/ α -agglutinin delivered into the surrounding culture fluid or being SDS extractable. In contrast to the laminarinase extraction of cell walls from cells expressing the free α -galactosidase, where no further liberation of any more enzyme was observed, identical treatment of fusion enzyme expressing cells released the overall bulk of the enzyme. This indicates that the fusion protein is intimately associated with the cell wall glucan in *S. cerevisiae*, like α -agglutinin, while α -galactosidase alone is not. The subsequently performed EndoH treatment showed a heavy glycosylation of the fusion protein, a result, entirely in agreement with the described extended glycosylation of the C-terminal part of α -agglutinin.

2. Localization

Immunofluorescent labelling with anti- α -galactosidase serum was performed on intact cells to determine the presence and distribution of α -galactosidase/ α -agglutinin fusion protein in the cell wall. Immunofluorescent labelling was carried out without fixing according to Watzel et al. (see reference 15). Cells of OD₅₃₀=2 were isolated and washed in TBS (10 mM Tris.HCl, pH 7.8, containing 140 mM NaCl, 5 mM EDTA and 20 μ g/ml cycloheximide). The cells were incubated in TBS+anti- α -galactosidase serum for 1 hour, followed by several washings in TBS. A subsequent incubation was carried out with FITC-conjugated anti-rabbit IgG (Sigma) for 30 minutes. After washing in TBS, cells were taken up in 10 mM Tris.HCl, pH 9.0, containing 1

mg/ml p-phenylenediamine and 0.1% azide and were photographed on a Zeiss 68000 microscope. The results of these analysis are given in FIG. 6, showing clearly that the chimeric α -galactosidase/ α -agglutinin is localized at the surface of the yeast cell. Buds of various sizes, even very small ones very uniformly labelled, demonstrates that the fusion enzyme is continuously incorporated into the cell wall throughout the cell cycle and that it instantly becomes tightly linked.

3. Activity

To quantitatively assay α -galactosidase activity, 200 μ l samples containing 0.1M sodium-acetate, pH 4.5 and 10 mM p-nitrophenyl- α -D-galactopyranoside (Sigma) were incubated at 37° C. for exactly 5 minutes. The reaction was stopped by addition of 1 ml 2% sodium carbonate. From intact cells and cell walls, removed by centrifugation and isolated and washed as described, the α -galactosidase activity was calculated using the extinction coefficient of p-nitrophenol of 18.4 cm²/μmole at 410 nm. One unit was defined as the hydrolysis of 1 μmole substrate per minute at 37° C.

TABLE 1

Distribution of free and immobilized α -galactosidase activity in yeast cells			
Expressed protein	α -Galactosidase activity (U/g F.W. cells)		
	Growth medium	Intact cells	Isolated cell walls
α -galactosidase	14.7	0.37	0.01
α Gal/ α AGG fusion protein	0.54	13.3	10.9

Transformed MT302/1C cells were in exponential phase (OD₅₃₀ = 2). One unit is defined as the hydrolysis of 1 μmole of p-nitrophenyl- α -D-galactopyranoside per minute at 37° C.

The results are summarized in Table 1. While the overall majority of α -galactosidase was distributed in the culture fluid, most of the fusion product was associated with the cells, primarily with the cell wall. Taking together the results shown in FIGS. 3 to 6 and in Table 1, it could be calculated that the enzymatic α -galactosidase activity of the chimeric enzyme is as good as that of the free enzyme. Moreover,

N.B. The essence of this EXAMPLE was published during the priority year by M. P. Schreuder et al. (see reference 25).

EXAMPLE 2A

Immobilized Humicola Lipase/ α -Agglutinin on the Surface of *S. cerevisiae*. (inducible expression of immobilized enzyme system)

The construction and isolation of the 1.4 kb *NheI*/*HindIII* fragment containing the C-terminal part of α -agglutinin has been described in EXAMPLE 1. Plasmid pUR7021 contains an 894 bp long synthetically produced DNA fragment encoding the lipase of *Humicola* (see reference 16 and SEQ ID NO: 7 and 8), cloned into the *EcoRI*/*HindIII* restriction sites of the commercially available vector pTZ18R (see FIG. 7). For the proper one-step modification of both the 5' end and the 3' end of the DNA part coding for the mature lipase, the PCR technique can be applied. Therefore the DNA oligonucleotides lipol (see SEQ ID NO: 3) and lipo2 (see SEQ ID NO: 6) can be used as primers in a standard PCR protocol, generating an 826 bp long DNA fragment with an *EagI* and a *HindIII* restriction site at the ends, which can be combined with the larger part of the *EagI*/*HindIII* digested pUR2650, a plasmid containing the α -galactosidase gene preceded by the invertase signal sequence as described earlier in this specification, thereby generating plasmid pUR2970A (see FIG. 7).

PCR oligonucleotides for the in-frame linkage of *Humicola* lipase and the C-terminus of α agglutinin.

a: PCR oligonucleotides for the transition between SUC2 signal sequence and the N-terminus of lipase.

```

                                >mature lipase
                                EagI  E  V  S  Q  D  L  F
primer lipol: 5'-GGG GCG GCC GAG GTC TCG CAA GAT CTG GA-3'
                                ||| ||| ||| ||| ||| ||| ||
lipase: 3'-TAA GCA GCT CTC CAG AGC GTT CTG GAC CTG TTT-5'
(non-coding strand, see SEQ ID NO: 4)

```

during stationary phase, the activity of the α -galactosidase in the growth medium decreased, whereas the activity of the cell wall associated α -galactosidase α -agglutinin fusion

b: PCR oligonucleotides for the in frame transition between C-terminus of lipase and C-terminal part of α -agglutinin.

```

                                F  G  L  I  G  T  C  L
lipase      5'-TTC GGG TTA ATT GGG ACA TGT CTT TAG TGC GA-3'
(cod. strand) ||| ||| ||| ||| ||| ||| ||| ||| ||
primer      3'-CCC AAT TAA CCC TGT ACA GAA CGA TCG GAA TTC GAACCC-5'
lipo2:      ||| ||| ||| ||| ||| ||| ||| ||| ||
(for the part of the lipase coding strand see SEQ ID NO: 5)
                                NheI  HindIII

```

remained constant, indicating that the cell associated fusion protein is protected from inactivation or proteolytic degradation.

Through the PCR method a *NheI* site will be created at the end of the coding sequence of the lipase, allowing the in-frame linkage between the DNA coding for lipase and the

DNA coding for the C-terminal part of α -agglutinin. Plasmid pUR2970A can then be digested with NheI and HindIII and the 1.4 kb NheI/HindIII fragment containing the C-terminal part of α -agglutinin from plasmid pUR2968 can be combined with the larger part of NheI and HindIII treated plasmid pUR2970A, resulting in plasmid pUR2971A. From this plasmid the 2.2 kb EagI/HindIII fragment can be isolated and ligated into the EagI- and HindIII-treated pUR2741, whereby plasmid pUR2741 is a derivative of pUR2740 (see reference 17), where the second EagI restriction site in the already inactive Tet resistance gene was deleted through NruI/SalI digestion. The SalI site was filled in prior to religation. The ligation then results in pUR2972A containing the GAL7 promoter, the invertase signal sequence, the chimeric lipase/ α -agglutinin gene, the 2 μ m sequence, the defective Leu2 promoter and the Leu2 gene. This plasmid can be used for transforming *S. cerevisiae* and the transformed cells can be cultivated in YP medium containing galactose as an inducer without repressing amounts of glucose being present, which causes the expression of the chimeric lipase/ α -agglutinin gene.

The expression, secretion, localization and activity of the chimeric lipase/ α -agglutinin can be analyzed using similar procedures as given in EXAMPLE 1.

In a similar way variants of Humicola lipase, obtained via rDNA techniques, can be linked to the C-terminal part of α -agglutinin, which variants can have a higher stability during (inter)esterification processes.

EXAMPLE 2B

Immobilized Humicola Lipase/ α -Agglutinin on the Surface of *S. cerevisiae* (inducible expression of immobilized enzyme system)

EXAMPLE 2A describes a protocol for preparing a particular construct. Before carrying out the work it was considered more convenient to use the expression vector described in EXAMPLE 1, so that the construction route given in this EXAMPLE 2B differs on minor points from the construction route given in EXAMPLE 2A and the resulting plasmids are not identical to those described in EXAMPLE 2A. However, the essential gene construct comprising the promoter, signal sequence, and the structural gene encoding the fusion protein are the same in EXAMPLES 2A and 2B.

1. Construction

The construction and isolation of the 1.4 kb NheI/HindIII fragment encoding the C-terminal part of α -agglutinin cell wall protein has been described in EXAMPLE 1. The plasmid pUR7033 (resembling pUR7021 of EXAMPLE 2A) was made by treating the commercially available vector pTZ18R with EcoRI and HindIII and ligating the resulting vector fragment with an 894 bp long synthetically produced DNA EcoRI/HindIII fragment encoding the lipase of Humicola (see SEQ ID NO: 7 and 8, and reference 16).

For the fusion of the lipase to the C-terminal, cell wall anchor-comprising domain of α -agglutinin, plasmid pUR7033 was digested with EagI and HindIII, and the lipase coding sequence was isolated and ligated into the EagI- and HindIII-digested yeast expression vector pSY1 (see reference 27), thereby generating pUR7034 (see FIG. 13). This is a 2 μ m episomal expression vector, containing the α -galactosidase gene described in EXAMPLE 1, preceded by the invertase (SUC2) signal sequence under the control of the inducible GAL7 promoter.

Parallel to this digestion, pUR7033 was also digested with EcoRV and HindIII, thereby releasing a 57 bp long DNA fragment, possessing codons for the last 15 carboxyterminal

amino acids. This fragment was exchanged against a small DNA fragment, generated through the hybridisation of the two chemically synthesized deoxyoligonucleotides SEQ ID NO: 9 and SEQ ID NO: 10. After annealing of both DNA strands, these two oligonucleotides essentially reconstruct the rest of the 3' coding sequence of the initial lipase gene, but additionally introduce downstream of the lipase gene a new NheI restriction site, followed by a HindIII site in close vicinity, whereby the first three nucleotides of the NheI site form the codon for the last amino acid of the lipase. The resulting plasmid was designated pUR2970B. Subsequently, this construction intermediate was digested with EagI and NheI, the lipase encoding fragment was isolated, and, together with the 1.4 kb NheI/HindIII fragment of pUR2968 ligated into the EagI- and HindIII-cut pSY1 vector. The outcome of this 3-point-ligation was called pUR2972B (see FIG. 14), the final lipase- α -agglutinin yeast expression vector.

This plasmid was used for transforming *S. cerevisiae* strain SU10 as described in reference 17 and the transformed cells were cultivated in YP medium containing galactose as the inducer without repressing amounts of glucose being present, which causes the expression of the chimeric lipase/ α -agglutinin gene.

2. Activity

To quantify the lipase activity, two activity measurements with two separate substrates were performed. In both cases, SU10 yeast cells transformed with either plasmid pUR7034 or pSY1 served as control. Therefore, yeast cell transformants containing either plasmid pSY1 or plasmid pUR7034 or plasmid pUR2972B were grown up for 24 h in YNB-glucose medium supplied with histidine and uracil, then diluted 1:10 in YP-medium supplied with 5% galactose, and again cultured. After 24 h incubation at 30° C., a first measurement for both assays was performed.

The first assay applied was the pH stat method. Within this assay, one unit of lipase activity is defined as the amount of enzyme capable of liberating one micromole of fatty acid per minute from a triglyceride substrate under standard assay conditions (30 ml assay solution containing 38 mM olive oil, considered as pure trioleate, emulsified with 1:1 w/w gum arabic, 20 mM calcium chloride, 40 mM sodium chloride, 5 mM Tris, pH 9.0, 30° C.) in a radiometer pH stat apparatus (pHM 84 pH meter, ABU 80 autoburette, TTA 60 titration assembly). The fatty acids formed were titrated with 0.05N NaOH and the activity measured was based on alkali consumption in the interval between 1 and 2 minutes after addition of putative enzyme batch. To test for immobilized lipase activity, 1 ml of each culture was centrifuged, the supernatant was saved, the pellet was resuspended and washed in 1 ml 1M sorbitol, subsequently again centrifuged and resuspended in 200 μ l 1M sorbitol. From each type of yeast cell the first supernatant and the washed cells were tested for lipase activity.

A: Lipase activity after 24 h (LU/ml)

	cell bound	culture fluid
pSY1	5.9	8.8
pUR7034	24.1	632.0
pUR2972B-(1)	18.7	59.6
pUR2972B-(2)	24.6	40.5

B: Lipase activity after 48 h (LU/ml)

	cell bound	culture fluid	OD660
pSY1	6.4	4.3	~40
pUR7034	215.0	2750.0	~40
pUR2972B-(1)	37.0	87.0	~40
pUR2972B-(2)	34.0	82.0	~40

The rest of the yeast cultures was further incubated, and essentially the same separation procedure was done after 48 hours. Dependent on the initial activity measured, the actual volume of the sample measured deviated between 25 μ l and 150 μ l.

This series of measurements indicates, that yeast cells comprising the plasmid coding for the lipase- α -agglutinin fusion protein in fact express some lipase activity which is associated with the yeast cell.

An additional second assay was performed to further confirm the immobilization of activity of lipase on the yeast cell surface. Briefly, within this assay, the kinetics of the PNP (=paranitrophenyl) release from PNP-butyrate is determined by measurement of the OD at 400 nm. Therefore, 10 ml cultures containing yeast cells with either pSY1, pUR7034 or pUR2972B were centrifuged, the pellet was resuspended in 4 ml of buffer A (0.1M NaOAc, pH 5.0 and 1 mM PMSF), from this 4 ml 500 μ l was centrifuged again and resuspended in 500 μ l PNB-buffer (20 mM Tris-HCl, pH 9.0, 20 mM CaCl₂, 25 mM NaCl), centrifuged once again, and finally resuspended in 400 μ l PNB buffer. This fraction was used to determine the cell bound fraction of lipase.

The remaining 3500 μ l were spun down, the pellet was resuspended in 4 ml A, to each of this, 40 μ l laminarinase (ex mollusc, 1.25 mU/ μ l) was added and first incubated for 3 hours at 37° C., followed by an overnight incubation at 20° C. Then the reaction mixture, still containing intact cells, were centrifuged again and the supernatant was used to determine the amount of originally cell wall bound material released through laminarinase incubation. The final pellet was resuspended in 400 μ l PNP buffer, to calculate the still cell associated part. The blank reaction of a defined volume of specific culture fraction in 4 ml assay buffer was determined, and then the reaction was started through addition of 80 μ l of substrate solution (100 mM PNP-butyrate in methanol), and the reaction was observed at 25° C. at 400 nm in a spectrophotometer.

	cell bound activity*	activity in the medium	laminarinase extract	laminarinase extracted cells	OD660
pSY1	0.001 (116 μ l)	0.001	0.028	0.000	2.6
pUR7034	0.293 (220 μ l)	0.446	0.076	0.985	2.36
pUR2972B-(1)	0.494 (143 μ l)	0.021	0.170	0.208	2.10

*unless otherwise mentioned, the volume of enzyme solution added was 20 μ l

This result positively demonstrates that a significant amount of lipase activity is immobilized on the surface yeast cell, containing plasmid pUR2972B. Here again, incorporation took place in such a way, that the reaction was catalyzed by cell wall inserted lipase of intact cells, indicated into the exterior orientated immobilization. Furthermore, the release of a significant amount of lipase activity after incubation with laminarinase again demonstrates the presumably covalent incorporation of a heterologous enzyme through gene fusion with the C-terminal part of α -agglutinin.

3. Localization

The expression, secretion, and subsequent incorporation of the lipase- α -agglutinin fusion protein into the yeast cell

wall was also confirmed through Immunofluorescent labeling with anti-lipase serum essentially as described in EXAMPLE 1, item 2. Localization.

As can be seen in FIG. 15, the Immunofluorescent stain shows essentially an analogous picture as the α -galactosidase immuno stain, with clearly detectable reactivity on the outside of the cell surface (see FIG. 15 A showing a clear halo around the cells and FIG. B showing a lighter circle at the surface of the cells), but neither in the medium nor in the interior of the cells. Yeast cells expressing pUR2972B, the Humicola lipase- α -agglutinin fusion protein, become homogeneously stained on the surface, indicating the virtually entire immobilization of a chimeric enzyme with an α -agglutinin C-terminus on the exterior of a yeast cell. In the performed control experiment SU10 yeast cells containing plasmid pUR7034 served as a control and here, no cell surface bound reactivity against the applied anti-lipase serum could be detected.

In a similar way variants of Humicola lipase, obtained via rDNA techniques, can be linked to the C-terminal part of α -agglutinin, which variants can have a higher stability during (inter)esterification processes.

EXAMPLE 3

Immobilized Humicola Lipase/ α -Agglutinin on the Surface of *S. cerevisiae* (constitutive expression of immobilized enzyme system)

Plasmid pUR2972 as described in EXAMPLE 2 can be treated with EagI and HindIII and the about 2.2 kb fragment containing the lipase/ α -agglutinin gene can be isolated. Plasmid pSY16 can be restricted with EagI and HindIII and between these sites the 2.2 kb fragment containing the lipase/ α -agglutinin fragment can be ligated resulting in pUR2973. The part of this plasmid that is involved in the production of the chimeric enzyme is similar to pUR2972 with the exception of the signal sequence. Whereas pUR2972 contains the SUC2-invertase-signal sequence, pUR2973 contains the α -mating factor signal sequence (see reference 18). Moreover the plasmid pUR2973 contains the Leu2 marker gene with the complete promoter sequence, instead of the truncated promoter version of pUR2972.

EXAMPLE 4

Immobilized Geotrichum Lipase/ α -Agglutinin on the Surface of *S. cerevisiae*

The construction and isolation of the 1.4 kb NheI/HindIII fragment comprising the C-terminal part of AG α -1

(α -agglutinin) gene has been described in EXAMPLE 1. For the in-frame gene fusion of the DNA coding for the C-terminal membrane anchor of α -agglutinin to the complete coding sequence of *Geotrichum candidum* lipase B from strain CMICC 335426 (see FIG. 8 and SEQ ID NO: 11 and 12), the plasmid pUR2974 can be used. This plasmid, derived from the commercially available pBluescript II SK plasmid, contains the cDNA coding for the complete *G. candidum* lipase II on an 1850 bp long EcoRI/XhoI insert (see FIG. 9).

To develop an expression vector for *S. cerevisiae* with homologous signal sequences, the N-terminus of the mature lipase B was determined experimentally by standard tech-

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niques. The obtained amino acid sequence of "Gln-Ala-Pro-Thr-Ala-Val..." is in complete agreement with the cleavage site of the signal peptidase on the *G. candidum* lipase II (see reference 19).

For the fusion of the mature lipase B to the *S. cerevisiae* signal sequences of SUC2 (invertase) or α -mating factor (prepro- α MF) on one hand and the in-frame fusion to the 3' part of the AG α 1 gene PCR technique can be used. The PCR primer lipo3 (see SEQ ID NO: 13) can be constructed in such a way, that the originally present EagI site in the 5'-part of the coding sequence (spanning codons 5-7 of the mature protein) will become inactivated without any alteration in the amino acid sequence. To facilitate the subsequent cloning procedures, the PCR primer can further contain a new EagI site at the 5' end, for the in-frame ligation to SUC2 signal sequence or prepro- α MF sequence, respectively. The corresponding PCR primer lipo4 (see SEQ ID NO: 16) contains an extra NheI site behind the nucleotides coding for the C-terminus of lipase B, to ensure the proper fusion to the C-terminal part of α -agglutinin.

PCR oligonucleotides for the in frame linkage of *G. candidum* lipase II to the SUC2 signal sequence and the C-terminal part of α -agglutinin.

a: N-terminal transition to either prepro α MF sequence or SUC2 signal sequence.

```

          EagI  A  Q  A  P  R  P  S  L  N
primer lipo3: 5'-GGG GCG GCG GCG CAG GCC CCA AGG CGG TCT CTC AAT-3'
              ||  ||| ||| ||| ||  ||| ||| ||| |||
lipaseII:      3'-GAC CGG GTC CGG GGT GCC GCC AGA GAG TTA-5'
(non-cod. strand, see SEQ ID NO: 14)

```

b: C-terminal fusion to C part of α -agglutinin

```

          S  N  F  E  T  D  V  N  L  Y  G
lipase:      5'-CA AAC TTT GAG ACT GAC GTT AAT CTC TAC GGT TAA AAC-3'
(cod. strand)
primer lipo4:      3'-C TGA CTG CAA TTA GAG ATG CCA CGATCG CCCC-5'
                      NheI
(for the part of the lipase coding strand see SEQ ID NO: 15)

```

The PCR product with the modified ends can be generated by standard PCR protocols, using instead of the normal Ampli-Taq polymerase the new thermostable VENT polymerase, which also exhibits proofreading activity, to ensure an error-free DNA template. Through digestion of the formerly described plasmid pUR2972 with EagI (complete) and NheI (partial), the Humicola lipase fragment can be exchanged against the DNA fragment coding for lipase B, thereby generating the final *S. cerevisiae* expression vector pUR2975 (see FIG. 9).

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The Humicola lipase- α -agglutinin fusion protein coding sequence can be exchanged against the lipase B/ α -agglutinin fusion construct described above by digestion of the described vector pUR2973 with EagI/HindIII, resulting in pUR2976 (see FIG. 9).

EXAMPLE 5

Immobilized *Rhizomucor miehei* Lipase/ α -Agglutinin on the Surface of *S. cerevisiae*

The construction and isolation of the 1.4 kb NheI/HindIII fragment encoding the C-terminal part of α -agglutinin has been described in EXAMPLE 1. The plasmid pUR2980 contains a 1.25 kb cDNA fragment cloned into the SmaI site of commercially available pUC18, which (synthetically synthesizable) fragment encodes the complete coding sequence of triglyceride lipase of *Rhizomucor miehei* (see reference 20), an enzyme used in a number of processes to interesterify triacylglycerols (see reference 21) or to prepare biosurfactants (see reference 22). Beside the 269 codons of the mature lipase molecule, the fragment also harbours codons for the 24 amino acid signal peptide as well as 70

amino acids of the propeptide. PCR can easily be applied to

ensure the proper fusion of the gene fragment encoding the mature lipase to the SUC2 signal sequence or the prepro α -mating factor sequence of *S. cerevisiae*, as well as the in-frame fusion to the described NheI/HindIII fragment. The following two primers, lipo5 (see SEQ ID NO: 17) and lipo6 (see SEQ ID NO: 20), will generate a 833 bp DNA fragment, which after Proteinase K treatment and digestion with EagI and NheI can be cloned as an 816 bp long fragment into the EagI/NheI digested plasmids pUR2972 and pUR2973, respectively (see FIG. 7).

```

          EagI  A  S  I  D  G  G  I
lipo5:      5'-CCC GCG GCC GCG AGC ATT GAT GGT GGT ATC-3'
              ||| ||| ||| ||| ||| |||
lipase (non-cod. strand):      3'-TCG TAA CTA GCA CCA TAG-5'
(for the part of the lipase non-coding strand, see SEQ ID NO: 18)

          N  T  G  L  C  T
lipase (cod. strand):      5'-AAC ACA GGC CTC TGT ACT-3'
              ||| ||| ||| ||| |||
Lipo6:      3'-TTG TGT CCG GAG ACA TGA CGATCGCGCC-5'
                      NheI
(for the part of the lipase coding strand, see SEQ ID NO: 19)

```

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These new *S. cerevisiae* expression plasmids contain the GAL7 promoter, the invertase signal sequence (pUR2981) or the prepro- α -mating factor sequence (pUR2982), the chimeric *Rhizomucor miehei* lipase/ α -agglutinin gene, the 2 μ m sequence, the defective (truncated) Leu2 promoter and the Leu2 gene. These plasmids can be transformed into *S. cerevisiae* and grown and analyzed using protocols described in earlier EXAMPLES.

EXAMPLE 6

Immobilized *Aspergillus niger* Glucose Oxidase/
GPI Anchored Cell Wall Proteins on the Surface of
S. cerevisiae

Glucose oxidase (β -D:oxygen 1-oxidoreductase, EC 1.1.3.4) from *Aspergillus niger* catalyses the oxidation of β -D-glucose to glucono- δ -lactone and the concomitant reduction of molecular oxygen to hydrogen peroxide. The fungal enzyme consists of a homodimer of molecular weight 150,000 containing two tightly bound FAD co-factors. Beside the use in glucose detection kits the enzyme is useful as a source of hydrogen peroxide in food preservation. The gene was cloned from both cDNA and genomic libraries, the single open reading frame contains no intervening sequences and encodes a protein of 605 amino acids (see reference 23).

With the help of two proper oligonucleotides the coding part of the sequence is adjusted in a one-step modifying procedure by PCR in such a way that a fusion gene product will be obtained coding for glucose oxidase and the C-terminal cell wall anchor of the FLO1 gene product or α -agglutinin. Thus, some of the plasmids described in former EXAMPLES can be utilized to integrate the corresponding sequence in-frame between one of the signal sequences used in the EXAMPLES and the NheI/HindIII part of the AGO1 gene.

Since dimerisation of the two monomers might be a prerequisite for activity, in an alternative approach the complete coding sequence for glucose oxidase without the GPI anchor can be expressed in *S. cerevisiae* transformant which already contains the fusion construct. This can be fulfilled by constitutive expression of the fusion construct containing the GPI anchor with the help of the GAPDH or PGK promoter for example. The unbound not-anchored monomer can be produced by using a DNA construct comprising an inducible promoter, as for instance the GAL7 promoter.

EXAMPLE 7

Process to Convert Raffinose, Stachyose and
Similar Sugars in Soy Extracts with α -
Galactosidase/ α -Agglutinin Immobilized on Yeasts

The yeast transformed with plasmid pUR2969 can be cultivated on large scale. At regular intervals during cultivation the washed cells should be analyzed on the presence of α -galactosidase activity on their surface with methods described in EXAMPLE 1. When both cell density and α -galactosidase activity/biomass reach their maximum, the yeast cells can then be collected by centrifugation and washed. The washed cells can then be added to soy extracts. The final concentration of the yeast cells can vary between 0.1 and 10 g/l, preferably the concentration should be above 1 g/l. The temperature of the soy extract should be $<8^{\circ}\text{C}$. to reduce the metabolic activity of the yeast cells. The conversion of raffinose and stachyose can be analyzed with HPLC methods and after 95% conversion of these sugars the yeasts

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cells can be removed by centrifugation and their α -galactosidase activity/g biomass can be measured. Centrifugates with a good activity can be used in a subsequent conversion process, whereas centrifugates with an activity of less than 50% of the original activity can be resuscitated in the growth medium and the cells can be allowed to recover for 2 to 4 hours. Thereafter the cells can be centrifuged, washed and subsequently be used in a subsequent conversion process.

EXAMPLE 8

Production of Biosurfactants Using *Humicola*
Lipase/ α -Agglutinin Immobilized on Yeasts.

The yeast transformed with plasmid pUR2972 or pUR2973 can be cultivated on large scale. At regular intervals during cultivation the washed cells can be analyzed on the presence of lipase activity on their surface with methods described in EXAMPLE 2. When both cell density and lipase/biomass reach their maximum, the yeast cells can be collected by centrifugation and washed. The washed cells can be suspended in a small amount of water and added to a reactor tank containing a mix of fatty acids, preferably of a chain length between 12-18 carbon atoms and sugars, preferably glucose, galactose or sucrose. The total concentration of the water (excluding the water in the yeast cells) might be below 0.1%. The final concentration of the yeast cells can vary between 0.1 and 10 g/l, preferably the concentration is above 1 g/l. The tank has to be kept under an atmosphere of N_2 and CO_2 in order to avoid oxidation of the (unsaturated) fatty acids and to minimize the metabolic activity of the yeasts. The temperature of mixture in the tank should be between $30-60^{\circ}\text{C}$., depending on type of fatty acid used. The conversion of fatty acids can be analyzed with GLC methods and after 95% conversion of these fatty acids the yeasts cells can be removed by centrifugation and their lipase activity/g biomass can be measured. Centrifugates with a good activity can be used in a subsequent conversion process, whereas centrifugates with an activity of less than 50% of the original activity can be resuscitated in the growth medium and the cells can be allowed to recover for 2 to 8 hours. Thereafter the cells can be centrifuged again, washed and used in a subsequent conversion process.

EXAMPLE 9

Production of Special Types of Triacylglycerols
using *Rhizomucor miehei* Lipase/ α -Agglutinin
Immobilized on Yeasts.

The yeast transformed with plasmid pUR2981 or pUR2982 can be cultivated on a large scale. At regular intervals during cultivation the washed cells can be analyzed on the presence of lipase activity on their surface with methods described in EXAMPLE 1. When both cell density and lipase/biomass reach their maximum, the yeast cells can be collected by centrifugation and washed. The washed cells can be suspended in a small amount of water and can be added to a reactor tank containing a mix of various triacylglycerols and fatty acids. The total concentration of the water (excluding the water in the yeast cells) might be below 0.1%. The final concentration of the yeast cells can vary between 0.1 and 10 g/l, preferably the concentration is above 1 g/l. The tank has to be kept under an atmosphere of N_2 and CO_2 in order to avoid oxidation of the (unsaturated) fatty acids and to minimize the metabolic activity of the yeasts. The temperature of mixture in the tank should be between

30–70° C., depending on types of triacylglycerol and fatty acid used. The degree of interesterification can be analyzed with GLC/MS methods and after formation of at least 80% of the theoretical value of the desired type of triacylglycerol the yeasts cells can be removed by centrifugation and their lipase activity/g biomass can be measured. Centrifugates with a good activity can be used in a subsequent conversion process, whereas centrifugates with an activity of less than 50% of the original activity is resuscitated in the growth medium and the cells should be allowed to recover 2 to 8 hours. After that the cells can be centrifuged, washed and used in a subsequent interesterification process.

Baker's yeasts of strain MT302/1C, transformed with either plasmid pSY13 or plasmid pUR2969 (described in EXAMPLE 1) were deposited under the Budapest Treaty at

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                    S   N   Y   A   V   S   T
primer pcrflol      5'- GAATTC GCT AGC AAT TAT GCT GTC AGT AAC - 3'
                        NheI   ||| ||| ||| ||| ||| |||
FL01 gene (non-coding strand)      3'- AGT TTA ATA CGA CAG TCA TGG TGA - 5'
(for the part of the non-coding strand, see SEQ ID NO: 24)

FL01 coding strand      5'-AATAA AATTCGCGTCTCTTTTACG - 3'
                        ||| ||| ||| ||| ||| ||| |||
primer pcrfl02:      3'-TTAAGCGCAAGAAAAATGC TTCGAACTCGAG - 5'
                                      HindIII
(for the part of the coding strand, see SEQ ID NO: 25)

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the Centraalbureau voor Schimmelcultures (CBS) on Jul. 3, 1992 under provisional numbers 330.92 and 329.92, respectively.

EXAMPLE 10

Immobilized Humicola Lipase/FLO1 Fusion on the Surface of *S. cerevisiae*

Flocculation, defined as "the (reversible) aggregation of dispersed yeast cells into flocs" (see reference 24), is the most important feature of yeast strains in industrial fermentations. Beside this it is of principal interest, because it is a property associated with cell wall proteins and it is a quantitative characteristic. One of the genes associated with the flocculation phenotype in *S. cerevisiae* is the FLO1 gene. The gene is located at approximately 24 kb from the right end of chromosome I and the DNA sequence of a clone containing major parts of FLO1 gene has very recently been determined (see reference 26). The sequence is given in FIG. 11 and SEQ ID NO: 21 and 22. The cloned fragment appeared to be approximately 2 kb shorter than the genomic copy as judged from Southern and Northern hybridizations, but encloses both ends of the FLO1 gene. Analysis of the DNA sequence data indicates that the putative protein contains at the N-terminus a hydrophobic region which confirms a signal sequence for secretion, a hydrophobic C-terminus that might function as a signal for the attachment of a GPI-anchor and many glycosylation sites, especially in the C-terminus, with 46.6% serine and threonine in the arbitrarily defined C-terminus (aa 271-894). Hence, it is likely that the FLO1 gene product is localized in an orientated fashion in the yeast cell wall and may be directly involved in the process of interaction with neighbouring cells. The cloned FLO1 sequence might therefore be suitable for the immobilization of proteins or peptides on the cell surface by a different type of cell wall anchor.

Recombinant DNA constructs can be obtained, for example by utilizing the DNA coding for amino acids 65 271-894 of the FLO1 gene product, i.e. polynucleotide 811-2682 of FIG. 11. Through application of two PCR

primers *perfl01* (see SEQ ID NO: 23) and *perfl02* (see SEQ ID NO: 26) *NheI* and *HindIII* sites can be introduced at both ends of the DNA fragment. In a second step, the 1.4 kb *NheI/HindIII* fragment present in pUR2972 (either A or B) containing the C-terminal part of α -agglutinin can be replaced by the 1.9 kb DNA fragment coding for the C-terminal part of the FLO1 protein, resulting in plasmid pUR2990 (see FIG. 12), comprising a DNA sequence encoding (a) the invertase signal sequence (SUC2) preceding (b) the fusion protein consisting of (b.1) the lipase of *Humicola* (see reference 16) followed by (b.2) the C-terminus of FLO1 protein (aa 271-894).

PCR oligonucleotides for the in frame connection of the genes encoding the Humicola lipase and the C-terminal part of the FLO1 gene product.

Plasmid pUR2972 (either A or B) can be restricted with *Nhe*I (partial) and *Hind*III and the *Nhe*I/*Hind*III fragment comprising the vector backbone and the lipase gene can be ligated to the correspondingly digested PCR product of the plasmid containing the FLO1 sequence, resulting in plasmid pUR2990, containing the GAL7 promoter, the *S. cerevisiae* invertase signal sequence, the chimeric lipase/FLO1 gene, the yeast 2 μ m sequence, the defective *Leu*2 promoter and the *Leu*2 gene. This plasmid can be transformed into *S. cerevisiae* and the transformed cells can be cultivated in YP medium including galactose as inducer.

The expression, secretion, localization and activity of the chimeric lipase/FLO1 protein can be analyzed using similar procedures as given in Example 1.

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SEQUENCE LISTING

(1) GENERAL INFORMATION:

(iii) NUMBER OF SEQUENCES: 26

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 6057 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(vi) ORIGINAL SOURCE:

- (A) ORGANISM: *Saccharomyces cerevisiae*

(ix) FEATURE:

- (A) NAME/KEY: CDS
- (B) LOCATION: 3653..5605
- (D) OTHER INFORMATION: /function= "sexual agglutination"
/product= "alpha-agglutinin"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

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Thr Gly Ser Ile Ser Thr Val Glu Thr Gly Asn Arg Thr Thr Ser Glu	
355 360 365	
GTG ATC AGT CAT GTG GTG ACT ACC AGC ACA AAA CTG TCT CCA ACT GCT	4807
Val Ile Ser His Val Val Thr Thr Ser Thr Lys Leu Ser Pro Thr Ala	
370 375 380 385	
ACT ACC AGC CTG ACA ATT GCA CAA ACC AGT ATC TAT TCT ACT GAC TCA	4855
Thr Thr Ser Leu Thr Ile Ala Gln Thr Ser Ile Tyr Ser Thr Asp Ser	
390 395 400	
AAT ATC ACA GTA GGA ACA GAT ATT CAC ACC ACA TCA GAA GTG ATT AGT	4903
Asn Ile Thr Val Gly Thr Asp Ile His Thr Thr Ser Glu Val Ile Ser	
405 410 415	
GAT GTG GAA ACC ATT AGC AGA GAA ACA GCT TCG ACC GTT GTA GCC GCT	4951
Asp Val Glu Thr Ile Ser Arg Glu Thr Ala Ser Thr Val Val Ala Ala	
420 425 430	
CCA ACC TCA ACA ACT GGA TGG ACA GGC GCT ATG AAT ACT TAC ATC CCG	4999
Pro Thr Ser Thr Thr Gly Trp Thr Gly Ala Met Asn Thr Tyr Ile Pro	
435 440 445	
CAA TTT ACA TCC TCT TCT TTC GCA ACA ATC AAC AGC ACA CCA ATA ATC	5047
Gln Phe Thr Ser Ser Phe Ala Thr Ile Asn Ser Thr Pro Ile Ile	
450 455 460 465	
TCT TCA TCA GCA GTA TTT GAA ACC TCA GAT GCT TCA ATT GTC AAT GTG	5095
Ser Ser Ser Ala Val Phe Glu Thr Ser Asp Ala Ser Ile Val Asn Val	
470 475 480	
CAC ACT GAA AAT ATC ACG AAT ACT GCT GCT GTT CCA TCT GAA GAG CCC	5143

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His Thr Glu Asn Ile Thr Asn Thr Ala Ala Val Pro Ser Glu Glu Pro	
485 490 495	
ACT TTT GTA AAT GCC ACG AGA AAC TCC TTA AAT TCC TTC TGC AGC AGC	5191
Thr Phe Val Asn Ala Thr Arg Asn Ser Leu Asn Ser Phe Cys Ser Ser	
500 505 510	
AAA CAG CCA TCC AGT CCC TCA TCT TAT ACG TCT TCC CCA CTC GTA TCG	5239
Lys Gln Pro Ser Ser Pro Ser Ser Tyr Thr Ser Ser Pro Leu Val Ser	
515 520 525	
TCC CTC TCC GTA AGC AAA ACA TTA CTA AGC ACC AGT TTT ACG CCT TCT	5287
Ser Leu Ser Val Ser Lys Thr Leu Leu Ser Thr Ser Phe Thr Pro Ser	
530 535 540 545	
GTG CCA ACA TCT AAT ACA TAT ATC AAA ACG GAA AAT ACG GGT TAC TTT	5335
Val Pro Thr Ser Asn Thr Tyr Ile Lys Thr Glu Asn Thr Gly Tyr Phe	
550 555 560	
GAG CAC ACG GCT TTG ACA ACA TCT TCA GTT GGC CTT AAT TCT TTT AGT	5383
Glu His Thr Ala Leu Thr Thr Ser Ser Val Gly Leu Asn Ser Phe Ser	
565 570 575	
GAA ACA GCA CTC TCA TCT CAG GGA ACG AAA ATT GAC ACC TTT TTA GTG	5431
Glu Thr Ala Leu Ser Ser Gln Gly Thr Lys Ile Asp Thr Phe Leu Val	
580 585 590	
TCA TCC TTG ATC GCA TAT CCT TCT TCT GCA TCA GGA AGC CAA TTG TCC	5479
Ser Ser Leu Ile Ala Tyr Pro Ser Ser Ala Ser Gly Ser Gln Leu Ser	
595 600 605	
GGT ATC CAA CAG AAT TTC ACA TCA ACT TCT CTC ATG ATT TCA ACC TAT	5527
Gly Ile Gln Gln Asn Phe Thr Ser Thr Ser Leu Met Ile Ser Thr Tyr	
610 615 620 625	
GAA GGT AAA GCG TCT ATA TTT TTC TCA GCT GAG CTC GGT TCG ATC ATT	5575
Glu Gly Lys Ala Ser Ile Phe Phe Ser Ala Glu Leu Gly Ser Ile Ile	
630 635 640	
TTT CTG CTT TTG TCG TAC CTG CTA TTC TAAACGGGT ACTGTACAGT	5622
Phe Leu Leu Ser Tyr Leu Leu Phe	
645 650	
TAGTACATTG AGTCGAAATA TACGAAATTA TTGTTCAATA TTTTCATCCT GGCTCTTTTT	5682
TTCTTCAACC ATAGTTAAAT GGACAGTTCA TATCTTAAAC TCTAATAATA CTTTCTAGT	5742
TCTTATCCTT TTCCGTCTCA CGCAGATTT TATCATAGTA TTAAATTIAT AITTTGTTTCG	5802
TAAAAAGAAA AATTGTGTGAG CGTTACCGCT CGTTTCATTA CCCGAAGGCT GTTTCAGTAG	5862
ACCACTGATT AAGTAAGTAG ATGAAAAAAT TTCATCACCA TGAAGAGTT CGATGAGAGC	5922
TACTTTTICA AATGCTTAAC AGCTAACCGC CATTCAATAA TGTTACGTTT TCTTCATTCT	5982
GCGGCTACGT TATCTAACAA GAGGTTTAC TCTCTCATAT CTCATTCAAA TAGAAAGAAC	6042
ATAATCAAAA AGCTT	6057

(2) INFORMATION FOR SEQ ID NO: 2:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 650 amino acids
 (B) TYPE: amino acid
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

Met Phe Thr Phe Leu Lys Ile Ile Leu Trp Leu Phe Ser Leu Ala Leu
 1 5 10 15

Ala Ser Ala Ile Asn Ile Asn Asp Ile Thr Phe Ser Asn Leu Glu Ile
 20 25 30

Thr Pro Leu Thr Ala Asn Lys Gln Pro Asp Gln Gly Trp Thr Ala Thr
 35 40 45

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Phe Asp Phe Ser Ile Ala Asp Ala Ser Ser Ile Arg Glu Gly Asp Glu
 50 55 60

Phe Thr Leu Ser Met Pro His Val Tyr Arg Ile Lys Leu Leu Asn Ser
 65 70 75 80

Ser Gln Thr Ala Thr Ile Ser Leu Ala Asp Gly Thr Glu Ala Phe Lys
 85 90 95

Cys Tyr Val Ser Gln Gln Ala Ala Tyr Leu Tyr Glu Asn Thr Thr Phe
 100 105 110

Thr Cys Thr Ala Gln Asn Asp Leu Ser Ser Tyr Asn Thr Ile Asp Gly
 115 120 125

Ser Ile Thr Phe Ser Leu Asn Phe Ser Asp Gly Gly Ser Ser Tyr Glu
 130 135 140

Tyr Glu Leu Glu Asn Ala Lys Phe Phe Lys Ser Gly Pro Met Leu Val
 145 150 155 160

Lys Leu Gly Asn Gln Met Ser Asp Val Val Asn Phe Asp Pro Ala Ala
 165 170 175

Phe Thr Glu Asn Val Phe His Ser Gly Arg Ser Thr Gly Tyr Gly Ser
 180 185 190

Phe Glu Ser Tyr His Leu Gly Met Tyr Cys Pro Asn Gly Tyr Phe Leu
 195 200 205

Gly Gly Thr Glu Lys Ile Asp Tyr Asp Ser Ser Asn Asn Asn Val Asp
 210 215 220

Leu Asp Cys Ser Ser Val Gln Val Tyr Ser Ser Asn Asp Phe Asn Asp
 225 230 235 240

Trp Trp Phe Pro Gln Ser Tyr Asn Asp Thr Asn Ala Asp Val Thr Cys
 245 250 255

Phe Gly Ser Asn Leu Trp Ile Thr Leu Asp Glu Lys Leu Tyr Asp Gly
 260 265 270

Glu Met Leu Trp Val Asn Ala Leu Gln Ser Leu Pro Ala Asn Val Asn
 275 280 285

Thr Ile Asp His Ala Leu Glu Phe Gln Tyr Thr Cys Leu Asp Thr Ile
 290 295 300

Ala Asn Thr Thr Tyr Ala Thr Gln Phe Ser Thr Thr Arg Glu Phe Ile
 305 310 315 320

Val Tyr Gln Gly Arg Asn Leu Gly Thr Ala Ser Ala Lys Ser Ser Phe
 325 330 335

Ile Ser Thr Thr Thr Thr Asp Leu Thr Ser Ile Asn Thr Ser Ala Tyr
 340 345 350

Ser Thr Gly Ser Ile Ser Thr Val Glu Thr Gly Asn Arg Thr Thr Ser
 355 360 365

Glu Val Ile Ser His Val Val Thr Thr Ser Thr Lys Leu Ser Pro Thr
 370 375 380

Ala Thr Thr Ser Leu Thr Ile Ala Gln Thr Ser Ile Tyr Ser Thr Asp
 385 390 395 400

Ser Asn Ile Thr Val Gly Thr Asp Ile His Thr Thr Ser Glu Val Ile
 405 410 415

Ser Asp Val Glu Thr Ile Ser Arg Glu Thr Ala Ser Thr Val Val Ala
 420 425 430

Ala Pro Thr Ser Thr Thr Gly Trp Thr Gly Ala Met Asn Thr Tyr Ile
 435 440 445

Pro Gln Phe Thr Ser Ser Ser Phe Ala Thr Ile Asn Ser Thr Pro Ile
 450 455 460

Ile Ser Ser Ser Ala Val Phe Glu Thr Ser Asp Ala Ser Ile Val Asn
 465 470 475 480

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Val His Thr Glu Asn Ile Thr Asn Thr Ala Ala Val Pro Ser Glu Glu
 485 490 495

Pro Thr Phe Val Asn Ala Thr Arg Asn Ser Leu Asn Ser Phe Cys Ser
 500 505 510

Ser Lys Gln Pro Ser Ser Pro Ser Ser Tyr Thr Ser Ser Pro Leu Val
 515 520 525

Ser Ser Leu Ser Val Ser Lys Thr Leu Leu Ser Thr Ser Phe Thr Pro
 530 535 540

Ser Val Pro Thr Ser Asn Thr Tyr Ile Lys Thr Glu Asn Thr Gly Tyr
 545 550 555 560

Phe Glu His Thr Ala Leu Thr Thr Ser Ser Val Gly Leu Asn Ser Phe
 565 570 575

Ser Glu Thr Ala Leu Ser Ser Gln Gly Thr Lys Ile Asp Thr Phe Leu
 580 585 590

Val Ser Ser Leu Ile Ala Tyr Pro Ser Ser Ala Ser Gly Ser Gln Leu
 595 600 605

Ser Gly Ile Gln Gln Asn Phe Thr Ser Thr Ser Leu Met Ile Ser Thr
 610 615 620

Tyr Glu Gly Lys Ala Ser Ile Phe Phe Ser Ala Glu Leu Gly Ser Ile
 625 630 635 640

Ile Phe Leu Leu Leu Ser Tyr Leu Leu Phe
 645 650

(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 29 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(vii) IMMEDIATE SOURCE:

- (B) CLONE: primer lipol

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

GGGGCGGCCG AGGTCTCGCA AGATCTGGA

29

(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 33 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(vii) IMMEDIATE SOURCE:

- (B) CLONE: Part non-coding strand lipase

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

TTTGTCAGG TCTGCGAGA CCTCTGACG AAT

33

(2) INFORMATION FOR SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 32 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

-continued

(vii) IMMEDIATE SOURCE:
(B) CLONE: Part coding strand lipase

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

TTCGGGTAA TTGGGACATG TCTTTAGTGC GA 32

(2) INFORMATION FOR SEQ ID NO: 6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 40 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(vii) IMMEDIATE SOURCE:
(B) CLONE: primer lipo2

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

CCCCAAGCTT AAGGCTAGCA AGACATGTCC CAATTAACCC 40

(2) INFORMATION FOR SEQ ID NO: 7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 894 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(vi) ORIGINAL SOURCE:
(A) ORGANISM: *Humicola lanuginosa*

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 72..884
(D) OTHER INFORMATION: /product= "lipase"

(ix) FEATURE:
(A) NAME/KEY: mat_peptide
(B) LOCATION: 72..881
(D) OTHER INFORMATION: /product= "lipase"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

GAATTCGTAG CGACGATATG AGGAGCTCCC TTGTGCTGTT CTTGTCTCT GCGTGGACGG 60

CCTTGGCCAC G GCC GAG GTC TCG CAA GAT CTG TTT AAC CAG TTC AAT CTC 110
Ala Glu Val Ser Gln Asp Leu Phe Asn Gln Phe Asn Leu
1 5 10

TTT GCA CAG TAT TCT GCT GCC GCA TAC TGC GGA AAA AAC AAT GAT GCC 158
Phe Ala Gln Tyr Ser Ala Ala Ala Tyr Cys Gly Lys Asn Asn Asp Ala
15 20 25

CCA GCT GGT ACA AAC ATT ACG TGC ACG GGA AAT GCC TGC CCC GAG GTA 206
Pro Ala Gly Thr Asn Ile Thr Cys Thr Gly Asn Ala Cys Pro Glu Val
30 35 40 45

GAG AAG GCG GAT GCA ACG TTT CTC TAC TCG TTT GAA GAC TCT GGA GTG 254
Glu Lys Ala Asp Ala Thr Phe Leu Tyr Ser Phe Glu Asp Ser Gly Val
50 55 60

GGC GAT GTC ACC GGC TTC CTT GCT CTA GAC AAC ACG AAC AAA TTG ATC 302
Gly Asp Val Thr Gly Phe Leu Ala Leu Asp Asn Thr Asn Lys Leu Ile
65 70 75

GTC CTC TCT TTC CGT GGC TCT CGT TCC ATA GAA AAC TGG ATC GGA AAT 350
Val Leu Ser Phe Arg Gly Ser Arg Ser Ile Glu Asn Trp Ile Gly Asn
80 85 90

CTT AAC TTC GAC TTG AAA GAA ATA AAT GAC ATT TGC TCC GGC TGC AGG 398
Leu Asn Phe Asp Leu Lys Glu Ile Asn Asp Ile Cys Ser Gly Cys Arg

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95	100	105	
GGA CAT GAC GGC TTC ACC TCG AGC TGG AGG TCT GTA GCC GAT ACG TTA Gly His Asp Gly Phe Thr Ser Ser Trp Arg Ser Val Ala Asp Thr Leu 110 115 120 125			446
AGG CAG AAG GTG GAG GAT GCT GTG AGG GAG CAT CCC GAC TAT CGC GTG Arg Gln Lys Val Glu Asp Ala Val Arg Glu His Pro Asp Tyr Arg Val 130 135 140			494
GTG TTT ACC GGA CAT AGC TTG GGT GGT GCA TTG GCA ACT GTT GCC GGA Val Phe Thr Gly His Ser Leu Gly Gly Ala Leu Ala Thr Val Ala Gly 145 150 155			542
GCA GAC CTG CGT GGA AAT GGG TAT GAC ATC GAC GTG TTT TCA TAT GGC Ala Asp Leu Arg Gly Asn Gly Tyr Asp Ile Asp Val Phe Ser Tyr Gly 160 165 170			590
GCC CCC CGA GTC GGA AAC AGG GCT TTT GCA GAA TTC CTG ACC GTA CAG Ala Pro Arg Val Gly Asn Arg Ala Phe Ala Glu Phe Leu Thr Val Gln 175 180 185			638
ACC GGC GGT ACC CTC TAC CGC ATT ACC CAC ACC AAT GAT ATT GTC CCT Thr Gly Gly Thr Leu Tyr Arg Ile Thr His Thr Asn Asp Ile Val Pro 190 195 200 205			686
AGA CTC CCG CCG CGC GAG TTC GGT TAC AGC CAT TCT AGC CCA GAG TAC Arg Leu Pro Pro Arg Glu Phe Gly Tyr Ser His Ser Ser Pro Glu Tyr 210 215 220			734
TGG ATC AAA TCT GGA ACC CTT GTC CCC GTC ACC CGA AAC GAC ATC GTG Trp Ile Lys Ser Gly Thr Leu Val Pro Val Thr Arg Asn Asp Ile Val 225 230 235			782
AAG ATA GAA GGC ATC GAT GCC ACC GGC GGC AAT AAC CAG CCT AAC ATT Lys Ile Glu Gly Ile Asp Ala Thr Gly Gly Asn Asn Gln Pro Asn Ile 240 245 250			830
CCG GAT ATC CCT GCG CAC CTA TGG TAC TTC GGG TTA ATT GGG ACA TGT Pro Asp Ile Pro Ala His Leu Trp Tyr Phe Gly Leu Ile Gly Thr Cys 255 260 265			878
CTT TAGTGCGAAG CTT Leu 270			894

(2) INFORMATION FOR SEQ ID NO: 8:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 270 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

Ala Glu Val Ser Gln Asp Leu Phe Asn Gln Phe Asn Leu Phe Ala Gln
1 5 10 15
Tyr Ser Ala Ala Ala Tyr Cys Gly Lys Asn Asn Asp Ala Pro Ala Gly
20 25 30
Thr Asn Ile Thr Cys Thr Gly Asn Ala Cys Pro Glu Val Glu Lys Ala
35 40 45
Asp Ala Thr Phe Leu Tyr Ser Phe Glu Asp Ser Gly Val Gly Asp Val
50 55 60
Thr Gly Phe Leu Ala Leu Asp Asn Thr Asn Lys Leu Ile Val Leu Ser
65 70 75 80
Phe Arg Gly Ser Arg Ser Ile Glu Asn Trp Ile Gly Asn Leu Asn Phe
85 90 95
Asp Leu Lys Glu Ile Asn Asp Ile Cys Ser Gly Cys Arg Gly His Asp
100 105 110
Gly Phe Thr Ser Ser Trp Arg Ser Val Ala Asp Thr Leu Arg Gln Lys

-continued

115	120	125
Val Glu Asp Ala Val Arg Glu His Pro Asp Tyr Arg Val Val Phe Thr 130	135	140
Gly His Ser Leu Gly Gly Ala Leu Ala Thr Val Ala Gly Ala Asp Leu 145	150	155
Arg Gly Asn Gly Tyr Asp Ile Asp Val Phe Ser Tyr Gly Ala Pro Arg 165	170	175
Val Gly Asn Arg Ala Phe Ala Glu Phe Leu Thr Val Gln Thr Gly Gly 180	185	190
Thr Leu Tyr Arg Ile Thr His Thr Asn Asp Ile Val Pro Arg Leu Pro 195	200	205
Pro Arg Glu Phe Gly Tyr Ser His Ser Ser Pro Glu Tyr Trp Ile Lys 210	215	220
Ser Gly Thr Leu Val Pro Val Thr Arg Asn Asp Ile Val Lys Ile Glu 225	230	235
Gly Ile Asp Ala Thr Gly Gly Asn Asn Gln Pro Asn Ile Pro Asp Ile 245	250	255
Pro Ala His Leu Trp Tyr Phe Gly Leu Ile Gly Thr Cys Leu 260	265	270

(2) INFORMATION FOR SEQ ID NO: 9:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 55 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

- (vii) IMMEDIATE SOURCE:
 - (B) CLONE: primer

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:

ATCCCTGCGC ACCTATGGTA CTTCGGGTGA ATTGGGACAT GTCTTGCTAG CCTTA

55

(2) INFORMATION FOR SEQ ID NO: 10:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 59 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

- (vii) IMMEDIATE SOURCE:
 - (B) CLONE: primer

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 10:

AGCTTAAGGC TAGCAAGACA TGTCCTCAATT AACCCGAAGT ACCATAGGTG CGCAGGGAT

59

(2) INFORMATION FOR SEQ ID NO: 11:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 1828 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: double
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA to mRNA.

- (vi) ORIGINAL SOURCE:
 - (A) ORGANISM: Geotrichum candidum
 - (B) STRAIN: CMICC 335426

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(ix) FEATURE:
 (A) NAME/KEY: CDS
 (B) LOCATION: 40..1731
 (D) OTHER INFORMATION: /product= "lipase"

(ix) FEATURE:
 (A) NAME/KEY: sig_peptide
 (B) LOCATION: 40..96

(ix) FEATURE:
 (A) NAME/KEY: mat_peptide
 (B) LOCATION: 97..1728
 (D) OTHER INFORMATION: /product= "lipase"
 /gene= "lipB"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 11:

AATTCGGCAC GAGATTCCTT TGATTGCAA CTGTTAATC ATG GTT TCC AAA AGC	54
Met Val Ser Lys Ser	
-19 -15	
TTT TTT TTG GCT GCG GCG CTC AAC GTA GTG GGC ACC TTG GCC CAG GCC	102
Phe Phe Leu Ala Ala Ala Leu Asn Val Val Gly Thr Leu Ala Gln Ala	
-10 -5 1	
CCC ACG GCC GTT CTT AAT GGC AAC GAG GTC ATC TCT GGT GTC CTT GAG	150
Pro Thr Ala Val Leu Asn Gly Asn Glu Val Ile Ser Gly Val Leu Glu	
5 10 15	
GGC AAG GTT GAT ACC TTC AAG GGA ATC CCA TTT GCT GAC CCT CCT GTT	198
Gly Lys Val Asp Thr Phe Lys Gly Ile Pro Phe Ala Asp Pro Pro Val	
20 25 30	
GGT GAC TTG CGG TTC AAG CAC CCC CAG CCT TTC ACT GGA TCC TAC CAG	246
Gly Asp Leu Arg Phe Lys His Pro Gln Pro Phe Thr Gly Ser Tyr Gln	
35 40 45 50	
GGT CTT AAG GCC AAC GAC TTC AGC TCT GCT TGT ATG CAG CTT GAT CCT	294
Gly Leu Lys Ala Asn Asp Phe Ser Ser Ala Cys Met Gln Leu Asp Pro	
55 60 65	
GGC AAT GCC TTT TCT TTG CTT GAC AAA GTA GTG GGC TTG GGA AAG ATT	342
Gly Asn Ala Phe Ser Leu Leu Asp Lys Val Val Gly Leu Gly Lys Ile	
70 75 80	
CTT CCT GAT AAC CTT AGA GGC CCT CTT TAT GAC ATG GCC CAG GGT AGT	390
Leu Pro Asp Asn Leu Arg Gly Pro Leu Tyr Asp Met Ala Gln Gly Ser	
85 90 95	
GTC TCC ATG AAT GAG GAC TGT CTC TAC CTT AAC GTT TTC CGC CCC GCT	438
Val Ser Met Asn Glu Asp Cys Leu Tyr Leu Asn Val Phe Arg Pro Ala	
100 105 110	
GGC ACC AAG CCT GAT GCT AAG CTC CCC GTC ATG GTT TGG ATT TAC GGT	486
Gly Thr Lys Pro Asp Ala Lys Leu Pro Val Met Val Trp Ile Tyr Gly	
115 120 125 130	
GGT GCC TTT GTG TTT GGT TCT TCT GCT TCT TAC CCT GGT AAC GGC TAC	534
Gly Ala Phe Val Phe Gly Ser Ser Ala Ser Tyr Pro Gly Asn Gly Tyr	
135 140 145	
GTC AAG GAG AGT GTG GAA ATG GGC CAG CCT GTT GTG TTT GTT TCC ATC	582
Val Lys Glu Ser Val Glu Met Gly Gln Pro Val Val Phe Val Ser Ile	
150 155 160	
AAC TAC CGT ACC GGC CCC TAT GGA TTC TTG GGT GGT GAT GCC ATC ACC	630
Asn Tyr Arg Thr Gly Pro Tyr Gly Phe Leu Gly Gly Asp Ala Ile Thr	
165 170 175	
GCT GAG GGC AAC ACC AAC GCT GGT CTG CAC GAC CAG CGC AAG GGT CTC	678
Ala Glu Gly Asn Thr Asn Ala Gly Leu His Asp Gln Arg Lys Gly Leu	
180 185 190	
GAG TGG GTT AGC GAC AAC ATT GCC AAC TTT GGT GGT GAT CCC GAC AAG	726
Glu Trp Val Ser Asp Asn Ile Ala Asn Phe Gly Gly Asp Pro Asp Lys	
195 200 205 210	
GTC ATG ATT TTC GGT GAG TCC GCT GGT GCC ATG AGT GTT GCT CAC CAG	774
Val Met Ile Phe Gly Glu Ser Ala Gly Ala Met Ser Val Ala His Gln	
215 220 225	

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CTT GTT GCC TAC GGT GGT GAC AAC ACC TAC AAC GGA AAG CAG CTT TTC Leu Val Ala Tyr Gly Gly Asp Asn Thr Tyr Asn Gly Lys Gln Leu Phe 230 235 240	822
CAC TCT GCC ATT CTT CAG TCT GGC GGT CCT CTT CCT TAC TTT GAC TCT His Ser Ala Ile Leu Gln Ser Gly Gly Pro Leu Pro Tyr Phe Asp Ser 245 250 255	870
ACT TCT GTT GGT CCC GAG AGT GCC TAC AGC AGA TTT GCT CAG TAT GCC Thr Ser Val Gly Pro Glu Ser Ala Tyr Ser Arg Phe Ala Gln Tyr Ala 260 265 270	918
GGA TGT GAC ACC AGT GCC AGT GAT AAT GAC ACT CTG GCT TGT CTC CGC Gly Cys Asp Thr Ser Ala Ser Asp Asn Asp Thr Leu Ala Cys Leu Arg 275 280 285 290	966
AGC AAG TCC AGC GAT GTC TTG CAC AGT GCG CAG AAC TCG TAT GAT CTT Ser Lys Ser Ser Asp Val Leu His Ser Ala Gln Asn Ser Tyr Asp Leu 295 300 305	1014
AAG GAC CTG TTT GGT CTG CTC CCT CAA TTC CTT GGA TTT GGT CCC AGA Lys Asp Leu Phe Gly Leu Leu Pro Gln Phe Leu Gly Phe Gly Pro Arg 310 315 320	1062
CCC GAC GGC AAC ATT ATT CCC GAT GCC GCT TAT GAG CTC TAC CGC AGC Pro Asp Gly Asn Ile Ile Pro Asp Ala Ala Tyr Glu Leu Tyr Arg Ser 325 330 335	1110
GGT AGA TAC GCC AAG GTT CCC TAC ATT ACT GGC AAC CAG GAG GAT GAG Gly Arg Tyr Ala Lys Val Pro Tyr Ile Thr Gly Asn Gln Glu Asp Glu 340 345 350	1158
GGT ACT ATT CTT GCC CCC GTT GCT ATT AAT GCT ACC ACT ACT CCC CAT Gly Thr Ile Leu Ala Pro Val Ala Ile Asn Ala Thr Thr Thr Pro His 355 360 365 370	1206
GTT AAG AAG TGG TTG AAG TAC ATT TGT AGC CAG GCT TCT GAC GCT TCG Val Lys Lys Trp Leu Lys Tyr Ile Cys Ser Gln Ala Ser Asp Ala Ser 375 380 385	1254
CTT GAT CGT GTT TTG TCG CTC TAC CCC GGC TCT TGG TCG GAG GGT TCA Leu Asp Arg Val Leu Ser Leu Tyr Pro Gly Ser Trp Ser Glu Gly Ser 390 395 400	1302
CCA TTC CGC ACT GGT ATT CTT AAT GCT CTT ACC CCT CAG TTC AAG CGC Pro Phe Arg Thr Gly Ile Leu Asn Ala Leu Thr Pro Gln Phe Lys Arg 405 410 415	1350
ATT GCT GCC ATT TTC ACT GAT TTG CTG TTC CAG TCT CCT CGT CGT GTT Ile Ala Ala Ile Phe Thr Asp Leu Leu Phe Gln Ser Pro Arg Arg Val 420 425 430	1398
ATG CTT AAC GCT ACC AAG GAC GTC AAC CGC TGG ACT TAC CTT GCC ACC Met Leu Asn Ala Thr Lys Asp Val Asn Arg Trp Thr Tyr Leu Ala Thr 435 440 445 450	1446
CAG CTC CAT AAC CTC GTT CCA TTT TTG GGT ACT TTC CAT GGC AGT GAT Gln Leu His Asn Leu Val Pro Phe Leu Gly Thr Phe His Gly Ser Asp 455 460 465	1494
CTT CTT TTT CAA TAC TAC GTG GAC CTT GGC CCA TCT TCT GCT TAC CGC Leu Leu Phe Gln Tyr Tyr Val Asp Leu Gly Pro Ser Ser Ala Tyr Arg 470 475 480	1542
CGC TAC TTT ATC TCG TTT GCC AAC CAC CAC GAC CCC AAC GTT GGT ACC Arg Tyr Phe Ile Ser Phe Ala Asn His His Asp Pro Asn Val Gly Thr 485 490 495	1590
AAC CTC CAA CAG TGG GAT ATG TAC ACT GAT GCA GGC AAG GAG ATG CTT Asn Leu Gln Gln Trp Asp Met Tyr Thr Asp Ala Gly Lys Glu Met Leu 500 505 510	1638
CAG ATT CAT ATG ATT GGT AAC TCT ATG AGA ACT GAC GAC TTT AGA ATC Gln Ile His Met Ile Gly Asn Ser Met Arg Thr Asp Asp Phe Arg Ile 515 520 525 530	1686
GAG GGA ATC TCG AAC TTT GAG TCT GAC GTT ACT CTC TTC GGT TAATCCCAT Glu Gly Ile Ser Asn Phe Glu Ser Asp Val Thr Leu Phe Gly 535 540 545	1738

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TAGCAAGTTT TGTGTATTTC AAGTATACCA GTTGATGTAA TATATCAATA GATTACAAAT 1798
 TAATTAGTGA AAAAAAAAAA AAAAAAAAAAC 1828

(2) INFORMATION FOR SEQ ID NO: 12:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 563 amino acids
 (B) TYPE: amino acid
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 12:

Met Val Ser Lys Ser Phe Phe Leu Ala Ala Ala Leu Asn Val Val Gly
 -19 -15 -10 -5
 Thr Leu Ala Gln Ala Pro Thr Ala Val Leu Asn Gly Asn Glu Val Ile
 1 5 10
 Ser Gly Val Leu Glu Gly Lys Val Asp Thr Phe Lys Gly Ile Pro Phe
 15 20 25
 Ala Asp Pro Pro Val Gly Asp Leu Arg Phe Lys His Pro Gln Pro Phe
 30 35 40 45
 Thr Gly Ser Tyr Gln Gly Leu Lys Ala Asn Asp Phe Ser Ser Ala Cys
 50 55 60
 Met Gln Leu Asp Pro Gly Asn Ala Phe Ser Leu Leu Asp Lys Val Val
 65 70 75
 Gly Leu Gly Lys Ile Leu Pro Asp Asn Leu Arg Gly Pro Leu Tyr Asp
 80 85 90
 Met Ala Gln Gly Ser Val Ser Met Asn Glu Asp Cys Leu Tyr Leu Asn
 95 100 105
 Val Phe Arg Pro Ala Gly Thr Lys Pro Asp Ala Lys Leu Pro Val Met
 110 115 120 125
 Val Trp Ile Tyr Gly Gly Ala Phe Val Phe Gly Ser Ser Ala Ser Tyr
 130 135 140
 Pro Gly Asn Gly Tyr Val Lys Glu Ser Val Glu Met Gly Gln Pro Val
 145 150 155
 Val Phe Val Ser Ile Asn Tyr Arg Thr Gly Pro Tyr Gly Phe Leu Gly
 160 165 170
 Gly Asp Ala Ile Thr Ala Glu Gly Asn Thr Asn Ala Gly Leu His Asp
 175 180 185
 Gln Arg Lys Gly Leu Glu Trp Val Ser Asp Asn Ile Ala Asn Phe Gly
 190 195 200 205
 Gly Asp Pro Asp Lys Val Met Ile Phe Gly Glu Ser Ala Gly Ala Met
 210 215 220
 Ser Val Ala His Gln Leu Val Ala Tyr Gly Gly Asp Asn Thr Tyr Asn
 225 230 235
 Gly Lys Gln Leu Phe His Ser Ala Ile Leu Gln Ser Gly Gly Pro Leu
 240 245 250
 Pro Tyr Phe Asp Ser Thr Ser Val Gly Pro Glu Ser Ala Tyr Ser Arg
 255 260 265
 Phe Ala Gln Tyr Ala Gly Cys Asp Thr Ser Ala Ser Asp Asn Asp Thr
 270 275 280 285
 Leu Ala Cys Leu Arg Ser Lys Ser Ser Asp Val Leu His Ser Ala Gln
 290 295 300
 Asn Ser Tyr Asp Leu Lys Asp Leu Phe Gly Leu Leu Pro Gln Phe Leu
 305 310 315

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Gly Phe Gly Pro Arg Pro Asp Gly Asn Ile Ile Pro Asp Ala Ala Tyr
 320 325 330

Glu Leu Tyr Arg Ser Gly Arg Tyr Ala Lys Val Pro Tyr Ile Thr Gly
 335 340 345

Asn Gln Glu Asp Glu Gly Thr Ile Leu Ala Pro Val Ala Ile Asn Ala
 350 355 360 365

Thr Thr Thr Pro His Val Lys Lys Trp Leu Lys Tyr Ile Cys Ser Gln
 370 375 380

Ala Ser Asp Ala Ser Leu Asp Arg Val Leu Ser Leu Tyr Pro Gly Ser
 385 390 395

Trp Ser Glu Gly Ser Pro Phe Arg Thr Gly Ile Leu Asn Ala Leu Thr
 400 405 410

Pro Gln Phe Lys Arg Ile Ala Ala Ile Phe Thr Asp Leu Leu Phe Gln
 415 420 425

Ser Pro Arg Arg Val Met Leu Asn Ala Thr Lys Asp Val Asn Arg Trp
 430 435 440 445

Thr Tyr Leu Ala Thr Gln Leu His Asn Leu Val Pro Phe Leu Gly Thr
 450 455 460

Phe His Gly Ser Asp Leu Leu Phe Gln Tyr Tyr Val Asp Leu Gly Pro
 465 470 475

Ser Ser Ala Tyr Arg Arg Tyr Phe Ile Ser Phe Ala Asn His His Asp
 480 485 490

Pro Asn Val Gly Thr Asn Leu Gln Gln Trp Asp Met Tyr Thr Asp Ala
 495 500 505

Gly Lys Glu Met Leu Gln Ile His Met Ile Gly Asn Ser Met Arg Thr
 510 515 520 525

Asp Asp Phe Arg Ile Glu Gly Ile Ser Asn Phe Glu Ser Asp Val Thr
 530 535 540

Leu Phe Gly

(2) INFORMATION FOR SEQ ID NO: 13:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 36 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(vii) IMMEDIATE SOURCE:
 (B) CLONE: primer lipo3

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 13:

GGGGCGGCCG CGCAGGCCCC AAGCGGTCT CTCAAT

36

(2) INFORMATION FOR SEQ ID NO: 14:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 30 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(vii) IMMEDIATE SOURCE:
 (B) CLONE: Part non-coding strand lipaseII

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 14:

ATTGAGAGAC CGCCGTGGGG CCTGGGCCAG

30

-continued

(2) INFORMATION FOR SEQ ID NO: 15:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 38 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(vii) IMMEDIATE SOURCE:

(B) CLONE: Part coding strand lipaseII

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 15:

CAAACCTTGA GACTGACGTT AATCTCTACG GTTAAAC

38

(2) INFORMATION FOR SEQ ID NO: 16:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 32 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(vii) IMMEDIATE SOURCE:

(B) CLONE: primer lipo4

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 16:

CCCCGCTAGC ACCGTAGAGA TTAACGTCAG TC

32

(2) INFORMATION FOR SEQ ID NO: 17:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 30 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(vii) IMMEDIATE SOURCE:

(B) CLONE: primer lipo5

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 17:

CCCGCGGCCG CGAGCATTGA TGGTGTATC

30

(2) INFORMATION FOR SEQ ID NO: 18:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 18 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(vii) IMMEDIATE SOURCE:

(B) CLONE: Part non-coding strand lipase

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 18:

GATACCACGA TCAATGCT

18

(2) INFORMATION FOR SEQ ID NO: 19:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 18 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

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(ii) MOLECULE TYPE: DNA (genomic)

(vii) IMMEDIATE SOURCE:
(B) CLONE: Part coding strand lipase

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 19:

AACACAGGCC TCTGTACT 18

(2) INFORMATION FOR SEQ ID NO: 20:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 28 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(vii) IMMEDIATE SOURCE:
(B) CLONE: primer lipo6

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 20:

CCGCGCTAGC AGTACAGAGG CCTGTGTT 28

(2) INFORMATION FOR SEQ ID NO: 21:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 2685 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) ORIGINAL SOURCE:
(A) ORGANISM: *Saccharomyces cerevisiae*

(vii) IMMEDIATE SOURCE:
(B) CLONE: pY105

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 1..2685
(D) OTHER INFORMATION: /product= "Flocculation protein" /gene= nFLO1"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 21:

ATG ACA ATG CCT CAT CGC TAT ATG TTT TTG GCA GTC TTT ACA CTT CTG	48
Met Thr Met Pro His Arg Tyr Met Phe Leu Ala Val Phe Thr Leu Leu	
1 5 10 15	
GCA CTA ACT AGT GTG GCC TCA GGA GCC ACA GAG GCG TGC TTA CCA GCA	96
Ala Leu Thr Ser Val Ala Ser Gly Ala Thr Glu Ala Cys Leu Pro Ala	
20 25 30	
GGC CAG AGG AAA AGT GGG ATG AAT ATA AAT TTT TAC CAG TAT TCA TTG	144
Gly Gln Arg Lys Ser Gly Met Asn Ile Asn Phe Tyr Gln Tyr Ser Leu	
35 40 45	
AAA GAT TCC TCC ACA TAT TCG AAT GCA GCA TAT ATG GCT TAT GGA TAT	192
Lys Asp Ser Ser Thr Tyr Ser Asn Ala Ala Tyr Met Ala Tyr Gly Tyr	
50 55 60	
GCC TCA AAA ACC AAA CTA GGT TCT GTC GGA GGA CAA ACT GAT ATC TCG	240
Ala Ser Lys Thr Lys Leu Gly Ser Val Gly Gly Gln Thr Asp Ile Ser	
65 70 75 80	
ATT GAT TAT AAT ATT CCC TGT GTT AGT TCA TCA GGC ACA TTT CCT TGT	288
Ile Asp Tyr Asn Ile Pro Cys Val Ser Ser Gly Thr Phe Pro Cys	
85 90 95	
CCT CAA GAA GAT TCC TAT GGA AAC TGG GGA TGC AAA GGA ATG GGT GCT	336
Pro Gln Glu Asp Ser Tyr Gly Asn Trp Gly Cys Lys Gly Met Gly Ala	
100 105 110	

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TGT TCT AAT AGT CAA GGA ATT GCA TAC TGG AGT ACT GAT TTA TTT GGT Cys Ser Asn Ser Gln Gly Ile Ala Tyr Trp Ser Thr Asp Leu Phe Gly 115 120 125	384
TTC TAT ACT ACC CCA ACA AAC GTA ACC CTA GAA ATG ACA GGT TAT TTT Phe Tyr Thr Thr Pro Thr Asn Val Thr Leu Glu Met Thr Gly Tyr Phe 130 135 140	432
TTA CCA CCA CAG ACG GGT TCT TAC ACA TTC AAG TTT GCT ACA GTT GAC Leu Pro Pro Gln Thr Gly Ser Tyr Thr Phe Lys Phe Ala Thr Val Asp 145 150 155 160	480
GAC TCT GCA ATT CTA TCA GTA GGT GGT GCA ACC GCG TTC AAC TGT TGT Asp Ser Ala Ile Leu Ser Val Gly Gly Ala Thr Ala Phe Asn Cys Cys 165 170 175	528
GCT CAA CAG CAA CCG CCG ATC ACA TCA ACG AAC TTT ACC ATT GAC GGT Ala Gln Gln Gln Pro Pro Ile Thr Ser Thr Asn Phe Thr Ile Asp Gly 180 185 190	576
ATC AAG CCA TGG GGT GGA AGT TTG CCA CCT AAT ATC GAA GGA ACC GTC Ile Lys Pro Trp Gly Gly Ser Leu Pro Pro Asn Ile Glu Gly Thr Val 195 200 205	624
TAT ATG TAC GCT GGC TAC TAT TAT CCA ATG AAG GTT GTT TAC TCG AAC Tyr Met Tyr Ala Gly Tyr Tyr Tyr Pro Met Lys Val Val Tyr Ser Asn 210 215 220	672
GCT GTT TCT TGG GGT ACA CTT CCA ATT AGT GTG ACA CTT CCA GAT GGT Ala Val Ser Trp Gly Thr Leu Pro Ile Ser Val Thr Leu Pro Asp Gly 225 230 235 240	720
ACC ACT GTA AGT GAT GAC TTC GAA GGG TAC GTC TAT TCC TTT GAC GAT Thr Thr Val Ser Asp Asp Phe Glu Gly Tyr Val Tyr Ser Phe Asp Asp 245 250 255	768
GAC CTA AGT CAA TCT AAC TGT ACT GTC CCT GAC CCT TCA AAT TAT GCT Asp Leu Ser Gln Ser Asn Cys Thr Val Pro Asp Pro Ser Asn Tyr Ala 260 265 270	816
GTC AGT ACC ACT ACA ACT ACA ACG GAA CCA TGG ACC GGT ACT TTC ACT Val Ser Thr Thr Thr Thr Thr Thr Glu Pro Trp Thr Gly Thr Phe Thr 275 280 285	864
TCT ACA TCT ACT GAA ATG ACC ACC GTC ACC GGT ACC AAC GGC GTT CCA Ser Thr Ser Thr Glu Met Thr Thr Val Thr Gly Thr Asn Gly Val Pro 290 295 300	912
ACT GAC GAA ACC GTC ATT GTC ATC AGA ACT CCA ACC AGT GAA GGT CTA Thr Asp Glu Thr Val Ile Val Ile Arg Thr Pro Thr Ser Glu Gly Leu 305 310 315 320	960
ATC AGC ACC ACC ACT GAA CCA TGG ACT GGC ACT TTC ACT TCG ACT TCC Ile Ser Thr Thr Thr Glu Pro Trp Thr Gly Thr Phe Thr Ser Thr Ser 325 330 335	1008
ACT GAG GTT ACC ACC ATC ACT GGA ACC AAC GGT CAA CCA ACT GAC GAA Thr Glu Val Thr Thr Ile Thr Gly Thr Asn Gly Gln Pro Thr Asp Glu 340 345 350	1056
ACT GTG ATT GTT ATC AGA ACT CCA ACC AGT GAA GGT CTA ATC AGC ACC Thr Val Ile Val Ile Arg Thr Pro Thr Ser Glu Gly Leu Ile Ser Thr 355 360 365	1104
ACC ACT GAA CCA TGG ACT GGT ACT TTC ACT TCT ACA TCT ACT GAA ATG Thr Thr Glu Pro Trp Thr Gly Thr Phe Thr Ser Thr Ser Thr Glu Met 370 375 380	1152
ACC ACC GTC ACC GGT ACT AAC GGT CAA CCA ACT GAC GAA ACC GTG ATT Thr Thr Val Thr Thr Thr Asn Gly Gln Pro Thr Asp Glu Thr Val Ile 385 390 395 400	1200
GTT ATC AGA ACT CCA ACC AGT GAA GGT TTG GTT ACA ACC ACC ACT GAA Val Ile Arg Thr Thr Thr Ser Glu Gly Leu Val Thr Thr Thr Thr Glu 405 410 415	1248
CCA TGG ACT GGT ACT TTT ACT TCG ACT TCC ACT GAA ATG TCT ACT GTC Pro Trp Thr Gly Thr Phe Thr Ser Thr Ser Thr Glu Met Ser Thr Val 420 425 430	1296

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ACT GGA ACC AAT GGC TTG CCA ACT GAT GAA ACT GTC ATT GTT GTC AAA Thr Gly Thr Asn Gly Leu Pro Thr Asp Glu Thr Val Ile Val Val Lys 435 440 445	1344
ACT CCA ACT ACT GCC ATC TCA TCC AGT TTG TCA TCA TCA TCT TCA GGA Thr Pro Thr Thr Ala Ile Ser Ser Ser Leu Ser Ser Ser Ser Gly 450 455 460	1392
CAA ATC ACC AGC TCT ATC ACG TCT TCG CGT CCA ATT ATT ACC CCA TTC Gln Ile Thr Ser Ser Ile Thr Ser Ser Arg Pro Ile Ile Thr Pro Phe 465 470 475 480	1440
TAT CCT AGC AAT GGA ACT TCT GTG ATT TCT TCC TCA GTA ATT TCT TCC Tyr Pro Ser Asn Gly Thr Ser Val Ile Ser Ser Ser Val Ile Ser Ser 485 490 495	1488
TCA GTC ACT TCT TCT CTA TTC ACT TCT TCT CCA GTC ATT TCT TCC TCA Ser Val Thr Ser Ser Leu Phe Thr Ser Ser Pro Val Ile Ser Ser Ser 500 505 510	1536
GTC ATT TCT TCT TCT ACA ACA ACC TCC ACT TCT ATA TTT TCT GAA TCA Val Ile Ser Ser Ser Thr Thr Thr Ser Thr Ser Ile Phe Ser Glu Ser 515 520 525	1584
TCT AAA TCA TCC GTC ATT CCA ACC AGT AGT TCC ACC TCT GGT TCT TCT Ser Lys Ser Ser Val Ile Pro Thr Ser Ser Ser Thr Ser Gly Ser Ser 530 535 540	1632
GAG AGC GAA ACG AGT TCA GCT GGT TCT GTC TCT TCT TCC TCT TTT ATC Glu Ser Glu Thr Ser Ser Ala Gly Ser Val Ser Ser Ser Ser Phe Ile 545 550 555 560	1680
TCT TCT GAA TCA TCA AAA TCT CCT ACA TAT TCT TCT TCA TCA TTA CCA Ser Ser Glu Ser Ser Lys Ser Pro Thr Tyr Ser Ser Ser Ser Leu Pro 565 570 575	1728
CTT GTT ACC AGT GCG ACA ACA AGC CAG GAA ACT GCT TCT TCA TTA CCA Leu Val Thr Ser Ala Thr Thr Ser Gln Glu Thr Ala Ser Ser Leu Pro 580 585 590	1776
CCT GCT ACC ACT ACA AAA ACG AGC GAA CAA ACC ACT TTG GTT ACC GTG Pro Ala Thr Thr Thr Lys Thr Ser Glu Gln Thr Thr Leu Val Thr Val 595 600 605	1824
ACA TCC TGC GAG TCT CAT GTG TGC ACT GAA TCC ATC TCC CCT GCG ATT Thr Ser Cys Glu Ser His Val Cys Thr Glu Ser Ile Ser Pro Ala Ile 610 615 620	1872
GTT TCC ACA GCT ACT GTT ACT GTT AGC GGC GTC ACA ACA GAG TAT ACC Val Ser Thr Ala Thr Val Thr Val Ser Gly Val Thr Thr Glu Tyr Thr 625 630 635 540	1920
ACA TGG TGC CCT ATT TCT ACT ACA GAG ACA ACA AAG CAA ACC AAA GGG Thr Trp Cys Pro Ile Ser Thr Thr Glu Thr Thr Lys Gln Thr Lys Gly 645 650 655	1968
ACA ACA GAG CAA ACC ACA GAA ACA ACA AAA CAA ACC ACG GTA GTT ACA Thr Thr Glu Gln Thr Thr Glu Thr Thr Lys Gln Thr Thr Val Val Thr 660 665 670	2016
ATT TCT TCT TGT GAA TCT GAC GTA TGC TCT AAG ACT GCT TCT CCA GCC Ile Ser Ser Cys Glu Ser Asp Val Cys Ser Lys Thr Ala Ser Pro Ala 675 680 685	2064
ATT GTA TCT ACA AGC ACT GCT ACT ATT AAC GGC GTT ACT ACA GAA TAC Ile Val Ser Thr Ser Thr Ala Thr Ile Asn Gly Val Thr Thr Glu Tyr 690 695 700	2112
ACA ACA TGG TGT CCT ATT TCC ACC ACA GAA TCG AGG CAA CAA ACA ACG Thr Thr Trp Cys Pro Ile Ser Thr Thr Glu Ser Arg Gln Gln Thr Thr 705 710 715 720	2160
CTA GTT ACT GTT ACT TCC TGC GAA TCT GGT GTG TGT TCC GAA ACT GCT Leu Val Thr Val Thr Ser Cys Glu Ser Gly Val Cys Ser Glu Thr Ala 725 730 735	2208
TCA CCT GCC ATT GTT TCG ACG GCC ACG GCT ACT GTG AAT GAT GTT GTT Ser Pro Ala Ile Val Ser Thr Ala Thr Ala Thr Val Asn Asp Val Val 740 745 750	2256

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ACG GTC TAT CCT ACA TGG AGG CCA CAG ACT GCG AAT GAA GAG TCT GTC Thr Val Tyr Pro Thr Trp Arg Pro Gln Thr Ala Asn Glu Glu Ser Val 755 760 765	2304
AGC TCT AAA ATG AAC AGT GCT ACC GGT GAG ACA ACA ACC AAT ACT TTA Ser Ser Lys Met Asn Ser Ala Thr Gly Glu Thr Thr Thr Asn Thr Leu 770 775 780	2352
GCT GCT GAA ACG ACT ACC AAT ACT GTA GCT GCT GAG ACG ATT ACC AAT Ala Ala Glu Thr Thr Thr Asn Thr Val Ala Ala Glu Thr Ile Thr Asn 785 790 795 800	2400
ACT GGA GCT GCT GAG ACG AAA ACA GTA GTC ACC TCT TCG CTT TCA AGA Thr Gly Ala Ala Glu Thr Lys Thr Val Val Thr Ser Ser Leu Ser Arg 805 810 815	2448
TCT AAT CAC GCT GAA ACA CAG ACG GCT TCC GCG ACC GAT GTG ATT GGT Ser Asn His Ala Glu Thr Gln Thr Ala Ser Ala Thr Asp Val Ile Gly 820 825 830	2496
CAC AGC AGT AGT GTT GTT TCT GTA TCC GAA ACT GGC AAC ACC AAG AGT His Ser Ser Ser Val Val Ser Val Ser Glu Thr Gly Asn Thr Lys Ser 835 840 845	2544
CTA ACA AGT TCC GGG TTG AGT ACT ATG TCG CAA CAG CCT CGT AGC ACA Leu Thr Ser Ser Gly Leu Ser Thr Met Ser Gln Gln Pro Arg Ser Thr 850 855 860	2592
CCA GCA AGC AGC ATG GTA GGA TAT AGT ACA GCT TCT TTA GAA ATT TCA Pro Ala Ser Ser Met Val Gly Tyr Ser Thr Ala Ser Leu Glu Ile Ser 865 870 875 880	2640
ACG TAT GCT GGC AGT GCA ACA GCT TAC TGG CCG GTA GTG GTT TAA Thr Tyr Ala Gly Ser Ala Thr Ala Tyr Trp Pro Val Val Val 885 890	2685

(2) INFORMATION FOR SEQ ID NO: 22:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 894 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 22:

Met Thr Met Pro His Arg Tyr Met Phe Leu Ala Val Phe Thr Leu Leu 1 5 10 15
Ala Leu Thr Ser Val Ala Ser Gly Ala Thr Glu Ala Cys Leu Pro Ala 20 25 30
Gly Gln Arg Lys Ser Gly Met Asn Ile Asn Phe Tyr Gln Tyr Ser Leu 35 40 45
Lys Asp Ser Ser Thr Tyr Ser Asn Ala Ala Tyr Met Ala Tyr Gly Tyr 50 55 60
Ala Ser Lys Thr Lys Leu Gly Ser Val Gly Gly Gln Thr Asp Ile Ser 65 70 75 80
Ile Asp Tyr Asn Ile Pro Cys Val Ser Ser Ser Gly Thr Phe Pro Cys 85 90 95
Pro Gln Glu Asp Ser Tyr Gly Asn Trp Gly Cys Lys Gly Met Gly Ala 100 105 110
Cys Ser Asn Ser Gln Gly Ile Ala Tyr Trp Ser Thr Asp Leu Phe Gly 115 120 125
Phe Tyr Thr Thr Pro Thr Asn Val Thr Leu Glu Met Thr Gly Tyr Phe 130 135 140
Leu Pro Pro Gln Thr Gly Ser Tyr Thr Phe Lys Phe Ala Thr Val Asp 145 150 155 160
Asp Ser Ala Ile Leu Ser Val Gly Gly Ala Thr Ala Phe Asn Cys Cys

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165				170				175						
Ala	Gln	Gln	Pro	Pro	Ile	Thr	Ser	Thr	Asn	Phe	Thr	Ile	Asp	Gly
	180						185					190		
Ile	Lys	Pro	Trp	Gly	Gly	Ser	Leu	Pro	Pro	Asn	Ile	Glu	Gly	Thr
	195						200					205		Val
Tyr	Met	Tyr	Ala	Gly	Tyr	Tyr	Pro	Met	Lys	Val	Val	Tyr	Ser	Asn
	210				215					220				
Ala	Val	Ser	Trp	Gly	Thr	Leu	Pro	Ile	Ser	Val	Thr	Leu	Pro	Asp
	225				230					235				240
Thr	Thr	Val	Ser	Asp	Asp	Phe	Glu	Gly	Tyr	Val	Tyr	Ser	Phe	Asp
			245						250				255	
Asp	Leu	Ser	Gln	Ser	Asn	Cys	Thr	Val	Pro	Asp	Pro	Ser	Asn	Tyr
		260						265					270	Ala
Val	Ser	Thr	Thr	Thr	Thr	Thr	Thr	Glu	Pro	Trp	Thr	Gly	Thr	Phe
		275						280				285		Thr
Ser	Thr	Ser	Thr	Glu	Met	Thr	Thr	Val	Thr	Gly	Thr	Asn	Gly	Val
	290					295				300				Pro
Thr	Asp	Glu	Thr	Val	Ile	Val	Ile	Arg	Thr	Pro	Thr	Ser	Glu	Gly
	305				310					315				320
Ile	Ser	Thr	Thr	Thr	Glu	Pro	Trp	Thr	Gly	Thr	Phe	Thr	Ser	Thr
		325						330					335	Ser
Thr	Glu	Val	Thr	Thr	Ile	Thr	Gly	Thr	Asn	Gly	Gln	Pro	Thr	Asp
		340					345					350		Glu
Thr	Val	Ile	Val	Ile	Arg	Thr	Pro	Thr	Ser	Glu	Gly	Leu	Ile	Ser
		355					360					365		Thr
Thr	Thr	Glu	Pro	Trp	Thr	Gly	Thr	Phe	Thr	Ser	Thr	Ser	Thr	Glu
	370					375				380				Met
Thr	Thr	Val	Thr	Gly	Thr	Asn	Gly	Gln	Pro	Thr	Asp	Glu	Thr	Val
	385					390				395				400
Val	Ile	Arg	Thr	Pro	Thr	Ser	Glu	Gly	Leu	Val	Thr	Thr	Thr	Glu
		405						410					415	
Pro	Trp	Thr	Gly	Thr	Phe	Thr	Ser	Thr	Ser	Thr	Glu	Met	Ser	Thr
		420						425				430		Val
Thr	Gly	Thr	Asn	Gly	Leu	Pro	Thr	Asp	Glu	Thr	Val	Ile	Val	Val
	435					440					445			Lys
Thr	Pro	Thr	Thr	Ala	Ile	Ser	Ser	Ser	Leu	Ser	Ser	Ser	Ser	Gly
	450					455				460				
Gln	Ile	Thr	Ser	Ser	Ile	Thr	Ser	Ser	Arg	Pro	Ile	Ile	Thr	Pro
	465				470					475				480
Tyr	Pro	Ser	Asn	Gly	Thr	Ser	Val	Ile	Ser	Ser	Ser	Val	Ile	Ser
		485						490				495		Ser
Ser	Val	Thr	Ser	Ser	Leu	Phe	Thr	Ser	Ser	Pro	Val	Ile	Ser	Ser
		500						505				510		Ser
Val	Ile	Ser	Ser	Ser	Thr	Thr	Thr	Ser	Thr	Ser	Ile	Phe	Ser	Glu
		515					520					525		Ser
Ser	Lys	Ser	Ser	Val	Ile	Pro	Thr	Ser	Ser	Ser	Thr	Ser	Gly	Ser
	530					535					540			Ser
Glu	Ser	Glu	Thr	Ser	Ser	Ala	Gly	Ser	Val	Ser	Ser	Ser	Ser	Phe
	545				550			555						560
Ser	Ser	Glu	Ser	Ser	Lys	Ser	Pro	Thr	Tyr	Ser	Ser	Ser	Ser	Leu
		565						570					575	Pro
Leu	Val	Thr	Ser	Ala	Thr	Thr	Ser	Gln	Glu	Thr	Ala	Ser	Ser	Leu
		580						585						590

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Pro Ala Thr Thr Thr Lys Thr Ser Glu Gln Thr Thr Leu Val Thr Val
 595 600 605
 Thr Ser Cys Glu Ser His Val Cys Thr Glu Ser Ile Ser Pro Ala Ile
 610 615 620
 Val Ser Thr Ala Thr Val Thr Val Ser Gly Val Thr Thr Glu Tyr Thr
 625 630 635 640
 Thr Trp Cys Pro Ile Ser Thr Thr Glu Thr Thr Lys Gln Thr Lys Gly
 645 650 655
 Thr Thr Glu Gln Thr Thr Glu Thr Thr Lys Gln Thr Thr Val Val Thr
 660 665 670
 Ile Ser Ser Cys Glu Ser Asp Val Cys Ser Lys Thr Ala Ser Pro Ala
 675 680 685
 Ile Val Ser Thr Ser Thr Ala Thr Ile Asn Gly Val Thr Thr Glu Tyr
 690 695 700
 Thr Thr Trp Cys Pro Ile Ser Thr Thr Glu Ser Arg Gln Gln Thr Thr
 705 710 715 720
 Leu Val Thr Val Thr Ser Cys Glu Ser Gly Val Cys Ser Glu Thr Ala
 725 730 735
 Ser Pro Ala Ile Val Ser Thr Ala Thr Ala Thr Val Asn Asp Val Val
 740 745 750
 Thr Val Tyr Pro Thr Trp Arg Pro Gln Thr Ala Asn Glu Glu Ser Val
 755 760 765
 Ser Ser Lys Met Asn Ser Ala Thr Gly Glu Thr Thr Thr Asn Thr Leu
 770 775 780
 Ala Ala Glu Thr Thr Thr Asn Thr Val Ala Ala Glu Thr Ile Thr Asn
 785 790 795 800
 Thr Gly Ala Ala Glu Thr Lys Thr Val Val Thr Ser Ser Leu Ser Arg
 805 810 815
 Ser Asn His Ala Glu Thr Gln Thr Ala Ser Ala Thr Asp Val Ile Gly
 820 825 830
 His Ser Ser Ser Val Val Ser Val Ser Glu Thr Gly Asn Thr Lys Ser
 835 840 845
 Leu Thr Ser Ser Gly Leu Ser Thr Met Ser Gln Gln Pro Arg Ser Thr
 850 855 860
 Pro Ala Ser Ser Met Val Gly Tyr Ser Thr Ala Ser Leu Glu Ile Ser
 865 870 875 880
 Thr Tyr Ala Gly Ser Ala Thr Ala Tyr Trp Pro Val Val Val
 885 890

(2) INFORMATION FOR SEQ ID NO: 23:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 30 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(vii) IMMEDIATE SOURCE:

- (B) CLONE: primer pcr101

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 23:

GAATTCGCTA GCAATTATGC TGTCAGTACC

30

(2) INFORMATION FOR SEQ ID NO: 24:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 24 base pairs

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(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(vii) IMMEDIATE SOURCE:
(B) CLONE: Part non-coding sequence FLO1

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 24:

AGTGGTACTG ACAGCATAAT TTGA

24

(2) INFORMATION FOR SEQ ID NO: 25:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(vii) IMMEDIATE SOURCE:
(B) CLONE: Part coding sequence FLO1

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 25:

AATAAATTC GCGTCTCTTT TACG

24

(2) INFORMATION FOR SEQ ID NO: 26:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 31 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(vii) IMMEDIATE SOURCE:
(B) CLONE: primer perflo2

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 26:

GAGCTCAAGC TTCGTAAAAA GAACGCGAAT T

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We claim:

1. A method for immobilizing an enzyme comprising recombinantly producing an enzyme or a functional fragment thereof linked to the exterior of a host cell, said method comprising localizing the enzyme or functional fragment thereof at the exterior of the cell wall of a fungus by linking the enzyme or the functional part thereof to the anchoring part of a cell wall anchoring protein, which anchoring part is derivable from the C-terminal part of said anchoring protein.

2. A method according to claim 1 in which said fungus is a yeast.

3. The method of claim 1, in which said fungus is selected from the group consisting of yeasts belonging to the genera *Candida*, *Debaryomyces*, *Hansenula*, *Kluyveromyces*, *Pichia* and *Saccharomyces*, and molds belonging to the genera *Aspergillus*, *Penicillium* and *Rhizopus*.

4. A fungus containing an expressible first polynucleotide comprising a structural gene encoding a protein providing catalytic activity and at least a part of a gene encoding at least a C-terminal portion of an anchoring protein capable of anchoring in the cell wall of said fungus, said part encoding at least the anchoring part of said anchoring protein, said first polynucleotide being present either in a vector or in the chromosome of said fungus.

5. The fungus of claim 4, further comprising a sequence encoding a signal peptide, said sequence being operably linked to said first polynucleotide such that the translation product of said first polynucleotide is secreted to the cell wall of said fungus.

6. The fungus of claim 5, wherein the signal peptide is derived from a protein selected from the group consisting of glycosyl-phosphatidyl-inositol (GPI) anchoring protein, α -factor, α -agglutinin, a-agglutinin, invertase or inulinase of yeasts, α -amylase of *Bacillus*, and proteinases of lactic acid bacteria.

7. The fungus of claim 4, wherein the protein capable of anchoring in the cell wall of said fungus is selected from the group consisting of α -agglutinin, a-agglutinin, flocculation protein, and Major Cell Wall Protein of a fungus.

8. The fungus of claim 4, wherein the protein providing catalytic activity is selected from the group consisting of a hydrolytic enzyme and an oxido-reductase.

9. The fungus of claim 8 wherein said hydrolytic enzyme is a lipase.

10. The fungus of claim 8 wherein said protein providing catalytic activity is an oxidase.

11. The fungus of claim 4, wherein the protein providing catalytic activity exhibits said catalytic activity when present in a multimeric form, said fungus further comprising a

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second polynucleotide comprising a structural gene encoding said protein providing catalytic activity operably linked to a sequence encoding a signal peptide ensuring secretion of the expression product of said second polynucleotide which is operably linked to a regulatable promoter.

12. The fungus of claim 11, wherein said second polynucleotide is present either in a separate vector than the first polynucleotide or is present in the chromosome of said fungus.

13. The fungus of claim 4, having at least one of said polynucleotides integrated in its chromosome.

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14. The fungus of claim 4, having said protein providing catalytic activity immobilized at the exterior of its cell wall.

15. The fungus of claim 4, which is a yeast.

16. A process for carrying out an enzymatic process by using an immobilized catalytically active protein, wherein a substrate for said catalytically active protein is contacted with the fungus of claim 4.

17. A process according to claim 16 in which the fungus is a yeast.

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